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MICROGLIA ACTIVATION IN A RODENT MODEL OF AN ALCOHOL USE DISORDER: THE IMPORTANCE OF PHENOTYPE, INITIATION, AND DURATION OF ACTIVATION

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
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By

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ABSTRACT

Chronic ethanol exposure results in neuroadaptations that drive the progression of an alcohol use disorder (AUD). One such driving force is alcohol-induced neurodegeneration. Neuroinflammation has been proposed as a mechanism underlying this damage. Although neuroinflammation is a physiological response to damage, overactivation of its pathways can lead to neurodegeneration. A hallmark indicator of neuroinflammation is microglial activation, but microglial activation is a heterogeneous continuum of phenotypes that can promote or inhibit neuroinflammation. Furthermore acute microglial activation is necessary to restore homeostasis, but prolonged activation can exacerbate damage. The diversity of microglia makes both the level and timecourse of activation vital to understanding their role in damage and/or recovery. The current set of experiments examines the effects of ethanol on microglia within the hippocampus and entorhinal cortex in a binge model of alcohol-induced neurodegeneration. In the first set of experiments, the phenotype of microglia activation was assessed using Raivich's 5-stages of activation that separates pro- and anti-inflammatory forms of microglia. Morphological and functional assessments suggest that ethanol does not elicit classical microglial activation but instead induces partially activated microglia. In the second set of experiments, the earliest signs of microglial activation were determined to understand the initiation of microglial activation. Experiments indicated that activation occurred subsequent to previous evidence of neuronal damage; however, activation was accompanied by a loss of microglia and the discovery of dystrophic microglia. The final set of experiments examined whether alcohol-induced partial activation of microglia would show a differential response with further alcohol exposure. Experiments showed that animals previously exposed to ethanol showed a greater response to a second ethanol insult. Overall, these studies suggest that although alcohol may initially interrupt the normal microglia response, during abstinence from ethanol a partial activation phenotype appears that may contribute to recovery. Once activated, however, data suggest that these microglia are primed and upon subsequent exposure show an increased response. This heterogeneous microglial response with respect to time does not necessarily reflect a neuroinflammatory response that would be neurodegenerative but does imply that chronic ethanol consumption affects the normal neuroimmune system.

KEYWORDS: microglia activation, alcohol use disorder, neurodegeneration, binge ethanol exposure, neuroinflammation

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LIST OF ABBREVIATIONS

APA- American Psychological Association
AUD-Alcohol Use Disorder
BBB- Blood Brain Barrier
BDNF- Brain Derived Neurotrophic Factor
CA- Cornu Amonis
CD- Cluster of Differentiation
CR3- Complement Receptor 3
CYP- CYtochrome P
CNS- Central Nervous System
DAB- 3,3'-DiAminoBenzidine tetrahydrochloride
DG- Dentate Gyrus
DSM- Diagnostic and Statistical Manual of mental disorders
ELISA- Enzyme Linked ImmunoSorbent Assay
ERCTX- EntoRhinal CorTex
EtOH- Ethanol
FASD- Fetal Alcohol Spectrum Disorders
FDA- Food and Drug Administration
GABA- Gamma-AminoButyric Acid
GFAP- Glial Fibrillary Acidic Protein
GLT-1- glial GLutame Transporter 1
GPCR- G Protein Coupled Receptor
ICD- International Classification of Diseases
IFNγ- InterFeroN-gamma
IL-6- InterLeukin-6
IL-10- InterLeukin-10
IP- IntraPeritoneal
LPS-LipoPolySaccharide
MCP-1- Monocyte Chemoattractant Protein-1
MCSF- Macrophage Colony Stimulating Factor
MHC-Major Histocompatibility Complex
MRI- Magnetic Resonance Imaging
nAChR- nicotinic Acetylcholine Receptor
NF-κB- Nuclear Factor kappa-light-chain-enhancer of activated B cells
NIH- National Institute of Health
NIAAA- National Institute on Alcohol Abuse and Alcoholism
NMDAR- N-Methyl-D-Aspartate Receptor
Poly IC- polyinosinic:polycytidylic acid
ROS- Reactive Oxygen Species
TBS- Tris Buffered Saline
TGF-β- Transforming Growth Factor-beta
TLR4- Toll Like Receptor 4
TNF-α- Tumor Necrosis Factor-alpha
TSPO- TranSlocator PrOtein

INTRODUCTION

Alcohol Use Disorders

Alcohol Use Disorders: Understanding the Problem

Alcohols are a group of organic compounds that have a hydroxyl functional group bound to a carbon atom. Due to ethanol's use in beverages, however, the two carbon chain alcohol has a notoriety that makes the term ethanol or ethyl alcohol (CH_3CH_2OH) synonymous with alcohol in the common vernacular. Consuming alcohol is a common socially accepted pastime, but habitual drunkenness or alcoholism is a problem that affects numerous aspects of society including but not limited to public health, the economy, and public safety (Schomerus et al. 2011). Alcoholism has officially been defined as a diagnosable medical condition or disease in the United States since 1980 with the production of the third edition of the Diagnostics and Statistics Manual of Mental Disorders (DSM; Hasin et al. 1996). Prior to the DSM III, alcoholism was only categorized as a personality disorder (Nathan 1991), but the World Health Organization removed alcoholism from a personality disorder in 1967 prior to its acceptance in the DSM (NIAAA 1995). The distinction between a personality disorder and a mental disease is important because the connotation of disease implies that alcoholism is more than just a behavioral problem and that treatment with pharmacotherapeutic interventions is appropriate (White et al. 2002).

The term alcoholism, although universally used to refer to habitual drunkenness, is actually an outdated term and was originally coined to refer to alcohol poisoning around 1850 by Magnus Huss, a Swedish professor of medicine (Lesch et al. 1990; Marcet 1860). In the DSM-IV, the American Psychological Association (APA) categorizes problems associated with alcohol misuse into two groups, alcohol abuse and alcohol dependence. Alcohol abuse and dependence fall under the umbrella term alcohol use

disorders (AUDs; American Psychiatric Association 2000). The two categories are defined by the same characteristics (Table 1.1), but alcohol abuse is defined as displaying one trait in a 12-month period whereas dependence requires possessing at least three traits within a 12-month period. These traits are very similar to other definitions of addiction including the development of tolerance, showing signs of withdrawal, and preoccupation with the drug of choice (American Psychiatric Association 2000). Other academic bodies such as WHO have similar definitions regarding alcohol abuse included in the International Classification of Diseases (ICD). The ICD differs in that it includes compulsivity as a characteristic which is absent in the DSM criteria, but both the DSM-IV and the ICD traits used to define AUDs have been validated in independent correlation studies predicting alcohol related problems within the general population (Grant et al. 2007). The development of these guidelines allows for diagnosis by clinicians, gives clear definitions for academic research, and most importantly, provides the general population with a way of understanding the boundaries between social and problematic drinking.

Table 1.1 Traits of Alcohol Abuse/Dependence

Traits	Characterized by:
Tolerance	▪ Increased amounts of alcohol required to achieve intoxication ▪ Diminished effects of the same amount of alcohol over time
Withdrawal	▪ Onset of characteristic withdrawal syndrome for alcohol including moderate symptoms like anxiety, and headache or more severe symptoms like seizure and fever. ▪ Drinking to relieve withdrawal symptoms
Impaired Control	▪ Persistent desire/unsuccessful efforts to curb drinking ▪ Drinking more or for a longer period than intended
Preoccupation	▪ Increased time spent in activities necessary to obtain/use alcohol or to recover from the effects of drinking
Continued Use Despite Problems	▪ Foregoing/reducing important social, occupational, or recreational activities because of drinking ▪ Ignoring persistent/recurrent physical or psychological problem that is likely to be caused or exacerbated by drinking

Table 1.1 Traits of an AUD as defined by the DSM-IV (Adapted from (American Psychiatric Association 2000; Hasin et al. 1996))

Despite these long standing institutional classifications, the societal debate whether alcoholism is a treatable disease or simply an inherent character flaw persists today (Schomerus et al. 2011). This debate continues in the face of overwhelming evidence showing altered brain function and structure in alcoholics, which suggest that habitual drunkenness or alcoholism fundamentally changes the neurobiology of individuals (Gunzerath et al. 2011). The perception that alcoholism is not a disease is slowly changing, but increasing the public and scientific communities' knowledge of the biological effects of alcoholism assists in advocacy efforts to accept alcoholism as a disease (Pescosolido et al. 2010). Furthermore, an understanding of alcoholism as a mental disease promotes treatment seeking by individuals who suffer from alcoholism as well as inclines counselors and clinicians to encourage the use of pharmacotherapies (Abraham et al. 2009; Schomerus et al. 2011).

Using the DSM- IV criteria for diagnosis (Table 1.1) , epidemiological studies show that in the United States AUDs are a common problem with over 8.5% of Americans fitting the diagnostic criteria within the last twelve months (Grant et al. 2004). This statistic is even more drastic when considering the lifetime prevalence of AUDs as almost 50% of men and nearly 25% of women at some point in their life could be diagnosed with having an AUD (Goldstein et al. 2012). This high prevalence makes AUDs a societal problem rather than just an individual issue. More than half of the United States population has a friend or close relative who currently has or previously suffered from an AUD (Dawson and Grant 1998). Problems associated with alcohol misuse are not unique to the United States of America as many other nations face similar issues with alcohol misuse (Bloomfield et al. 2003; Grittner et al. 2012). Even individuals who choose to completely abstain from alcohol consumption and are fortunate enough to not have direct social ties to anyone with an AUD are still affected

by the rampant use and abuse of alcohol. For example, in 2006, problems associated with alcohol cost the United States approximately $223.5 billion meaning that it cost each individual approximately $746 per year regardless of their choice to abstain or drink (Bouchery et al. 2011). This exorbitant amount includes costs associated with lost productivity, healthcare issues, criminal justice procedures, property damage, and many other contributing factors (Bouchery et al. 2011).

The majority of these problems stem from binge drinking (Bouchery et al. 2011). Binge drinking is defined as five or more drinks for a male or four or more drinks for a female in a two hour period that results in a blood ethanol concentration (BEC) of at least 0.08% (NIAAA 2004). A drink consists of one-half US fluid ounces of pure ethanol which roughly equates to a bottle of beer, one mixed drink, or a glass of wine (Miller et al. 1991). Clear definitions of what constitutes both a drink and the behavioral outcomes associated with a particular number of drinks are vital for the public to be able to predict intoxication behaviors and consider the associated consequences. Fortunately, the popular media as well as colleges across the nation understand the prevalence of alcohol abuse and have published this data in multiple formats for the general population (McCoppin 2012; O'Callaghan 2009); however, people continue to underestimate the size of a drink, which results in higher alcohol consumption than intended and therefore higher BECs (White et al. 2003).

The pattern of drinking is crucial as it is a better predictor of both BECs and the associated problematic outcomes than the type of drink or the lifetime quantity of drinking (Bobak et al. 2004). For example, although an individual who drinks a nightly shot of alcohol will consume the same lifetime quantity of alcohol as someone who abstains during the week but chooses to drink seven beers every Saturday night, the individual drinking seven beers has a greater likelihood of suffering consequences from

the acute effects of intoxication due to higher BECs. These consequences are varied but include things like poor decision-making (George et al. 2005), risky sexual behavior (Stappenbeck et al. 2013), and a tendency to be involved in violent crimes as either the perpetrator or the victim (Boles and Miotto 2003). Table 1.2 shows the effects of different BECs on intoxication behaviors that underlie the consequences of acute alcohol intoxication. For example, impaired judgment at BECs above 50mg/dL would be associated with poor decision-making. The legal limit in most states 80mg/dL was set based on the relationship between BEC and consequces such as the impairments in motor function seen at above a 0.08 that can lead to increased vehicular accidents (Villaveces et al. 2003; Whetten-Goldstein et al. 2000). Although other factors can influence BECs such as sex, genetic differences in alcohol metabolism, and body mass index (Koob and Le Moal 2006), the number of drinks is the most common predictor used to understand intoxication and has been shown to be associated with the greater risks and alcohol-related problems (Fillmore and Jude 2011; Koob and Le Moal 2006).

Table 1.2 Outcomes Associated with Different BECs

BEC (mg/dL)	BEC (%)	Number of Drinks	Outcomes
10-50	0.01-0.05	1-2	Increased sociability; talkativeness; disinhibition; anxiolytic; euphoria
50-150	0.05-0.15	2-5	Significant disinhibition; impaired judgment cognitive and motor function, sedation
150-200	0.15-0.20	5-6	Major motor impairments; slurred speech; delayed reaction time
200-300	0.20- 0.30	6-9	Hypnotic effects; stuporous but conscious behavior
300+	0.30+	9+	Anesthesia; coma; death

Table 1.2 Outcomes associated with various BECs and the number of drinks to achieve the range for a 140 pound male. The second column shows the more commonly used blood alcohol percent by law enforcement and the media (Adapted from (Koob and Le Moal 2006))

Although acute intoxication may lead to some of the societal problems from alcohol misuse, continual episodes of excessive drinking and its corresponding high BECs results in more permanent changes to organ systems. These biological effects caused by alcohol are considered causative in at least 30 diseases and may make individuals more susceptible to countless others (Rehm 2011; Room et al. 2005). Moreover, these biological changes promote the development of dependence by creating traits like tolerance through alterations of alcohol metabolism or the perception of the drug rewarding effect of alcohol in the central nervous system (CNS; Djordjevic et al. 1998; Gilpin and Koob 2008).

Alcohol Use Disorders: Alcohol as a Reward

The euphoria associated with a drug or the "high" is one of the central mechanisms by which drugs, including ethanol, are rewarding (Koob and Le Moal 2001). This high occurs due to changes in neurotransmission particularly in the "feel good" neurotransmitters, dopamine and serotonin (Gilpin and Koob 2008). The rewarding affects of alcohol can occur at low concentrations as shown in Table 1.2 (Boileau et al. 2003). However, repetitive use of drugs of abuse can cause neuroplastic changes that alter the normal hedonic systems of neurotransmission (Der-Avakian and Markou 2012). In prolonged periods without the drug in the system, the neuroplastic changes lead to a feeling of dysphoria or a "low" leading to a sense of craving for the drug (Der-Avakian and Markou 2012; Markou et al. 1998).

Alcohol can also be rewarding due to its anxiolytic effects (Wallner et al. 2003; Wallner et al. 2006). Many people consume ethanol for its soothing effects, but similar to chronic ethanol's effects on hedonic systems, repeated exposure alters the neurotransmitter systems associated with anxiety causing neuroplastic changes that produce hyperexcitability (Engin et al. 2012). Addiction, in this instance AUDs, is

therefore driven by both the rewarding (euphoric and anxiolytic) effects of intoxication and the agitating (dysphoric and anxiogenic) effects caused by ethanol (Koob and Le Moal 2001; Koob and Volkow 2010; Lingford-Hughes et al. 2010). This introduction will cover how neurodegeneration from chronic alcohol exposure can affect cognition and indirectly affect the rewarding characteristics of alcohol as these studies are intended to find novel targets to reduced alcohol brain damage. However, the current AUD therapies have direct actions on ethanol reward by altering neurotransmitter systems and will be discussed first.

Alcohol Use Disorders: Ethanol Pharmacology

The rewarding effects of alcohol are due to ethanol's neuropharmacological actions, but chronic use alters these systems such that the absence of the drug leads to dysphoria (Clapp et al. 2008). While some drugs of abuse are known to bind to a specific receptor disrupting hedonic pathways of neurotransmission, alcohol pharmacology is more complex due to its physical and chemical properties (Vengeliene et al. 2008). For example, one theory is that ethanol, as a small, amphiphilic molecule, can displace water preferentially due to its attraction to both hydrophobic and hydrophilic targets (Klemm 1998). The displacement of water can then affect the confirmation state of receptors making alcohol an allosteric effector of various neurotransmitter systems (Klemm 1990). The multitude of ethanol effects on various neurobiological substrates makes a full review of alcohol pharmacology within this dissertation impossible; therefore, this brief synopsis will focus on the major effects of ethanol's rewarding effects by discussing the cholinergic and opioid systems as it relates to euphoria as well as the balance of GABAergic and glutamatergic systems as it relates to the anxiolytic effects of alcohol. Furthermore, these systems were chosen because the majority of the current

pharmacotherapy interventions for AUDs or alcohol withdrawal target these systems. Each system and the respective drug that targets the system will be discussed briefly.

Cholinergic System & Varenicline

Acute ethanol can change the binding of endogenous acetylcholine, particularly to the nicotinic acetylcholine receptors (nAChR; Cardoso et al. 1999; Zuo et al. 2004). nAChR's are ionotropic receptors consisting of a single, pentameric transmembrane channel. The composition of the pentamer alters the alcohol effects on nAChR (Cardoso et al. 1999; Narahashi et al. 1999; Zuo et al. 2002). At low concentrations, alcohol acts as co-agonist enhancing cholinergic binding in receptors with α_2 and α_4 subunits (Marszalec et al. 1999). This increased ligand binding and ethanol's ability to stabilize the open channel state enhances the influx of Na^+ depolarizing cells, increases the probability of an action potential propagation, and results in the increased release of neurotransmitters (Forman and Zhou 1999). Acute ethanol's effects on the cholinergic system, particularly on the $\alpha_4\beta_2$ subtype, can therefore increase dopaminergic signaling and create a sense of euphoria (Blomqvist et al. 2002; Borghese et al. 2003; McGranahan et al. 2011). The transient, increased dopamine concentration created by ethanol's action on nAChR is only one way that alcohol is rewarding (Soderpalm et al. 2009).

Because of ethanol's effects on the cholinergic system, varenicline, a partial agonist of the $\alpha_4\beta_2$ nAChR subtype, has been proposed as a drug of interest for AUDs. Varenicline can reduce alcohol consumption and seeking but is not yet approved by the US Food and Drug Administration (FDA) for the treatment of AUDs (Mitchell et al. 2012; Steensland et al. 2007). Its actions are thought to be associated with reduced euphoria caused by acute alcohol intoxication through the reduction of the cholinergic effects on dopamine release (Ericson et al. 2009; Hendrickson et al. 2010).

Opioid System & Naltrexone

The euphoria associated with ethanol use is not only caused by alcohol's action on the cholinergic system but can also be linked to its effects on the opioid system (Vengeliene et al. 2008). The endogenous opioid system consists of a group of G protein-coupled receptors (GPCR's) and their associated ligands like dynorphins, enkephalins, and endorphins (Bodnar 2012). Acute ethanol stimulates the release of opioid peptides, especially β-endorphin (Gianoulakis 2001; Warren and Hewitt 2010). This increase in endogenous opioids has a euphoric effect due to the opiate systems relationship to the dopaminergic system (Gianoulakis 2001; Spanagel et al. 1990). However, the effects of chronic ethanol exposure are not as clear, but some reports indicate alcohol increases μ-opioid receptors as a compensatory mechanism of chronic alcohol's effect to reduce the binding of endogenous opioids to receptors (Djouma and Lawrence 2002).

The effects of ethanol on the opioid system mean that manipulating the system affects the rewarding capacity of alcohol. Naltrexone is a μ-opioid receptor antagonist that is indicated for AUD treatment by the FDA. The efficacy of naltrexone is due to its ability to antagonize the μ-opioid receptor and therefore reduce the effects of alcohol-induced increased β-endorphin (Littleton and Zieglgansberger 2003; Ray et al. 2010). The reduction in endogenous opiates reduces the ethanol-induced dopaminergic increase and euphoric effects of alcohol (Kato 2008; Valenta et al. 2013). Naltrexone has shown some efficacy in AUD treatments by increasing drug abstinence and/or reducing the number of drinks (Lee et al. 2012; Pettinati et al. 2011).

Glutamatergic System & Acamprosate

As stated earlier, ethanol can also be rewarding outside of the euphoric high due to its sedative or anxiolytic effects. Some of the anxiolytic effects are associated with ethanol's pharmacological actions within the glutamate system, the major excitatory

system (Tsai and Coyle 1998). Although alcohol can affect various types of glutamate receptors, studies on the effects of alcohol on the glutamatergic system generally focus on the *N*-methyl-D-aspartate receptor (NMDAR). The focus on the NMDAR stems from research showing that low doses of alcohol inhibit NMDA-activated Ca^{2+} calcium influx (Lovinger et al. 1989). The reduction in excitation from ethanol's effects on the NMDAR system underlies the anxiolytic effects of acute alcohol exposure (Tsai and Coyle 1998). Chronic exposure, however, causes upregulation of the NMDARs on the cell surface of neurons (Sheela Rani and Ticku 2006). This upregulation of NMDARs can cause a state of hyperexcitation when ethanol is acutely withdrawn and is associated with severe withdrawal symptoms like seizures and convulsions (Hoffman 1995; Tsai and Coyle 1998). Moreover, the neuroplastic changes induced by chronic ethanol exposure in NMDAR activity and expression make the system more sensitive to glutamate (Vengeliene et al. 2008). Alcohol's effects on the glutamatergic system will be revisited later in this dissertation as it also considered a source of neuronal damage.

Acamprosate is an FDA approved therapy that targets the effects of ethanol on the glutamatergic system. It is the most readily prescribed treatment for alcoholism although its use is still relatively low (Mark et al. 2009). As a weak partial agonist of NMDAR, acamprosate reduces the dysphoria in alcohol withdrawal by attenuating hyper- glutamatergic signaling (Mann et al. 2008; Umhau et al. 2010). A reduction in the negative affects associated with alcohol deprivation afforded by acamprosate is thought to reduce relapse and make it useful in the treatment of AUDs (Heilig and Egli 2006).

GABAergic System & Benzodiazepines

Ethanol not only depresses the major excitatory neurotransmitter system, but also enhances the transmission of the major inhibitory neurotransmitter, γ-aminobutyric acid (GABA; Vengeliene et al. 2008). Ethanol's allosteric effects on the $GABA_A$ receptor

in conjunction with its glutamatergic effects play a large role in the anxiolytic state associated alcohol (Koob 2004; Tsai and Coyle 1998). Like the other neurotransmitter systems, alcohol has the ability to affect various subtypes of GABA receptors, but it has particular actions on the $GABA_A$ receptor (Kumar et al. 2009; Lobo and Harris 2008). $GABA_A$ receptors are ligand gated ion channels but, being inhibitory, are responsible for the efflux of the anion Cl^- when activated (Spitzer 2010). Low doses of ethanol, increase the binding of GABA to its receptor through an allosteric mechanism, but electrophysiology studies have shown that high, but physiologically relevant concentrations of ethanol can also have direct effects on the GABA receptor in the absence of GABA (Aguayo et al. 2002). Regardless of whether alcohol allosterically alters the receptor or directly acts as a ligand, the anion influx hyperpolarizes neurons so that there is a reduction in synaptic transmission. The depressed synaptic transmission state is associated with the rewarding, sedative hypnotic properties of alcohol (Koob 2004). However, chronic ethanol exposure causes an internalization of GABA receptors and reduces the ability of agonists to bind (Golovko et al. 2002). The resulting reduced GABAergic tone is thought to lead to a state of hyperexcitability and anxiogenic effects of alcohol withdrawal (Golovko et al. 2002; Koob 2004).

To reduce this hyperexcitation state, benzodiazepines, $GABA_A$ agonists, have been used effectively for years to reduce acute alcohol withdrawal symptoms (Doble 1999; Mayo-Smith 1997; Ntais et al. 2005). However, benzodiazepines are not approved as an FDA treatment for AUDs as they ameliorate acute withdrawal effects but do not necessarily affect the rewarding effects of alcohol (Ntais et al. 2005). Although alcohol causes neuroplastic changes in GABAergic system, benzodiazepines, unlike the aforementioned medications, are not effective in reducing alcohol intake because they do not change the actual perception of alcohol but rather treat a symptom.

Disulfiram

Not all drugs used for AUD therapy have been based on changes in neurobiology. Disulfiram is also an FDA approved drug for AUDs whose action is mainly based on inhibiting the metabolism of alcohol within the liver. Disulfiram inhibition of the enzyme acetaldehyde dehydrogenase causes a buildup of an ethanol metabolite, acetaldehyde (Jorgensen et al. 2011). Increases in acetaldehyde cause nausea, headaches, and various other negative reactions so that drinking ethanol produces an immediate aversive effect (Barth and Malcolm 2010). Although disulfiram has been shown to have some neurobiological effects on reward and craving (Barth and Malcolm 2010; Grant and Dawson 1998), its aversive nature makes patient compliance and therefore clinical utility within AUDs problematic (Jorgensen et al. 2011). Like benzodiazepines, disulfiram's main effects are not on the addictive effects of alcohol; however, varenicline, naltrexone, and acamprosate use within AUD therapy are great examples of how determining chronic ethanol's neuroplastic changes associated with the progression of addiction led to treatment options (Chou et al. 1998). Unfortunately, the efficacy of these drugs in the general population is still low and suggests that alternatives therapeutic targets need to be discovered.

Alcohol Use Disorders: Neurodegeneration & Cognitive Deficits

Not only does excessive alcohol consumption alter functional aspects of the brain like neurotransmission that can drive AUD development, it can also result in more global structural changes through cellular damage (Crews and Nixon 2009; Harper 2009). The use of therapies that target the neuroadaptations in neurotransmission caused by chronic ethanol exposure suggests that, as another neuroplastic change, alcohol-induced neurodegeneration may also be a potential target for AUD therapies. Currently, no FDA approved drug specifically targets alcohol-induced brain damage (Wang et al.

2010). Although alcohol-induced neurodegeneration can be associated with thiamine deficiency, alcoholic brain damage, as discussed herein, will refer only to damage independent of nutritional deficiency and not Wernicke-Korsakoff's syndrome (Thomson et al. 2012). Furthermore, the focus will be on brain damage that occurs from AUDs as opposed to the neuronal loss that may endure from prenatal exposure as seen in fetal alcohol spectrum disorders (FASD; Klintsova et al. 2007; Lewis et al. 2012; West and Goodlett 1990).

Scientific debates regarding alcohol-induced brain damage have a long history but were initially based on deficits in cognition seen in alcoholics due to methodological limitations (Freund 1973; Freund and Walker 1971). The first quantitative study looking at alcohol related brain damage in humans showed a reduction in weights of alcoholic individuals' brains compared to social drinkers (Harper and Blumbergs 1982). However, with the advent of new techniques such as magnetic resonance imaging (MRI), studies have been able to show more specific brain regions within alcoholics that have reduced volume compared with moderate, social drinkers (Pfefferbaum et al. 1992; Pfefferbaum et al. 1995; Sullivan et al. 1995; Zahr et al. 2011). This damage includes a loss of both cortical grey and white matter resulting in thinner gyri and increased sulci and lateral ventricles in alcoholics (Mann et al. 2001; Pfefferbaum et al. 1992; Pfefferbaum et al. 1995). Post-mortem examinations of the brains of alcoholics concur with MRI findings showing reductions in volume and/or neuronal cell number of various regions (Agartz et al. 1999; Phillips et al. 1987). These regions include the cerebellum (Baker 1999; Phillips et al. 1987; Sullivan et al. 2010), hippocampus (Agartz et al. 1999; Beresford et al. 2006; Sullivan et al. 1995), corpus callosum (Pfefferbaum 1996; Pfefferbaum and Sullivan 2002), and cortical regions especially the frontal lobe (Pfefferbaum et al. 1992; Pfefferbaum et al. 1997). Others, however, have not seen differences in the

hippocampus of alcoholics (Harper 1998), but the majority of studies indicate volume loss or damage . It is important to denote that neurodegeneration does not necessarily occur in all individuals that drink, but instead, alcohol-induced brain damage correlates with chronic, excessive ethanol consumption levels and particularly binge drinking (Hunt 1993; Lisdahl et al. 2013).

Although neurodegeneration is not directly related to the rewarding effects of alcohol pharmacologically, neuronal damage can indirectly affect feelings of reward as well as alter other behavioral attributes associated with AUD development and addiction (Crews and Boettiger 2009; Kelley and Mittleman 1999; Koob and Le Moal 1997). Neurodegeneration within a specific brain region as well as damage to the integrity of its circuits can be correlated to decline in behaviors associated with that region (Alfonso-Loeches and Guerri 2011; Zahr et al. 2011). For example, damage seen in the frontal cortex of the mesocorticolimbic pathway has been associated with poor executive function (Bechara 2005; Dawson and Grant 1998; Medina et al. 2008; Pfefferbaum et al. 1997). Damage to this region caused by ethanol is also thought to be the cause of increased impulsivity observed in alcoholics in tasks like delayed discounting procedures (Crews and Boettiger 2009; Petry 2001). Decreased executive function and increased impulsivity leads to the poor decision making concerning alcohol and may be one of the reasons that alcoholics have problems with drug preoccupation (Crews 2008; Gilpin and Koob 2008; Parsons 1993).

Neurodegeneration-induced behavioral deficits can promote a “spiral of addiction” (Crews 1999; Koob and Le Moal 1997). The spiral of addiction involves the promotion of alcohol intake by various factors that influence one another in a cyclical pattern. For example, an individual who starts off drinking moderate volumes of ethanol may progressively consume more ethanol due to drug tolerance. Tolerance to ethanol can

develop from various biological changes including increased drug metabolism (Djordjevic et al. 1998), altered alcohol neuropharmacology (Vengeliene et al. 2008), and behavioral adaptations that allow for normal functioning during intoxication (Vogel-Sprott 1997). Regardless of the type of tolerance, the result is increased ethanol consumption which can cause neurodegeneration in the frontal lobes (Pfefferbaum et al. 1992; Sullivan et al. 1995). Neurodegeneration then compromises the ability of the alcoholic individual to make good decisions regarding ethanol consumption (Crews et al. 2005). This theory highlights how the structural neuroplastic changes in just one region of the brain caused by excessive alcohol consumption can promote alcohol abuse.

Herein, the focus will be on neurodegeneration within the hippocampus and the entorhinal cortex. The hippocampus is important for learning and memory and has been postulated to have a role in drug addiction by its control of drug-dependent memories and influence on the prefrontal cortex (Hyman et al. 2006; Nixon et al. 2011). The hippocampus is connected with frontal cortical regions by glutamatergic efferent neurons, so hippocampal damage can also affect behaviors associated with the frontal lobe (Godsil et al. 2013). Because the entorhinal cortex and the hippocampus are highly interconnected (Burwell and Amaral 1998), damage to in the entorhinal cortex also affects hippocampal integrity and contributes to cognitive deficits (Bott et al. 2013; Harich et al. 2008). The hippocampus and entorhinal cortex were chosen as compromised hippocampal integrity in alcoholics has been proposed to underlie behavioral deficits observed in executive function as well as working memory (Beresford et al. 2006; Chanraud et al. 2010). Furthermore, the model used in this dissertation has repeatedly produced damage in both the entorhinal cortex and the hippocampus across many labs (Collins et al. 1996; Kelso et al. 2011; Obernier et al. 2002a).

Alcohol Use Disorders: Models of Alcohol-Induced Neurodegeneration

Many models of alcohol exposure exist due to the complex nature of the contributing factors of AUD progression. People drink for a variety of reasons; therefore, no single model is enough to fully understand alcoholism. Herein, only the subset of *in vivo* rodent models that produce alcohol-induced neurodegeneration are discussed, but reviews are available that discuss models that examine other aspects of alcoholism such as the rewarding effects and the behavioral effects of intoxication (Crabbe et al. 2011; Ripley and Stephens 2011). Models that specifically elicit alcohol-induced neurodegeneration are necessary in order to understand the mechanisms that lead to brain damage in alcoholics and can therefore participate in the progression of AUDs by contributing to the spiral of addiction previously described. Unfortunately, most animals do not voluntarily consume ethanol at the high concentrations associated with neurodegeneration. Some labs circumvent this problem by using *in vitro* studies that expose neuronal and/or glial tissue cultures to different concentrations and durations of ethanol that cause neuronal damage or evidence of stress (Fernandez-Lizarbe et al. 2009; Prendergast et al. 2004). However, animal models have to use forced ethanol exposure to study the phenomenon of alcohol-induced neuronal loss (Crews et al. 2004; Crews and Nixon 2009). These categories fall under four basic categories of ethanol exposure: injections, vapor exposure, chronic feeding, or intragastric gavage. Importantly, these models mimic damage in brain regions and the cognitive deficits seen in alcoholic patients. Intragastric gavage will be the method used throughout the work presented herein but other methods will be discussed briefly subsequently.

Intraperitoneal (ip) injections of ethanol can produce evidence of neurodegeneration. In studies using this model, animals received 3g/kg of ethanol via ip injections for two consecutive days with two day gaps without injections over a two week or month long period (Lundqvist et al. 1995; Pascual et al. 2007). This intermittent

pattern over at least a two week period causes damage in the cerebellum, hippocampus, and neocortex (Lundqvist et al. 1995; Pascual et al. 2007). Neurodegeneration in this particular model appears to be dependent upon repeated cycling of high BECs and withdrawal phases (Lundqvist et al. 1995; Lundqvist et al. 1994). This neuronal damage also causes cognitive problems including persistent alterations in the hippocampal associated task of object recognition (Barker and Warburton 2011; Pascual et al. 2007). The ip route of ethanol administration is problematic as it does not necessarily mimic alcohol kinetics associated with the typical oral route of alcohol administration in the human population which makes translating results from these studies difficult (Adalsteinsson et al. 2006; Iwaniec and Turner 2013).

Similar to the ip injections, there are problems with the face validity associated of alcohol vapor inhalation models of AUDs (Mattucci-Schiavone and Ferko 1986; Ripley and Stephens 2011). Although there have been recent reports in the popular media of a trend of alcohol inhalation (Sifferlin 2013), drinking oral ethanol is still by far the most common route of intoxication. Vapor studies vary on the dose, duration, and pattern of ethanol exposure (Gilpin et al. 2008), but the derivation with chronic intermittent vapor exposure has shown evidence of neurodegeneration in the hippocampus (Ehlers et al. 2013). The vapor model of alcohol-induced neurodegeneration not only alters the cognitive abilities directly associated with brain damage but also has behavioral correlates associated with other traits of alcoholism including increased self-administration (Gilpin et al. 2009; O'Dell et al. 2004). Despite having some behavioral attributes of an AUD, bypassing the normal metabolic pathways of ethanol makes the vapor inhalation models problematic as inhaling alcohol produces different behavioral outcomes than oral administration (Mattucci-Schiavone and Ferko 1986).

The last two model types that will be discussed have better face validity in that they both at least use an oral route of ethanol administration which is most similar to the human condition (Bell et al. 2012). Chronic feeding models rely on self-administration over months whereas the intragastric gavage uses forced intubation over a relatively short timeline. In the chronic feeding models, ethanol is the only source of fluid in drinking water but not food (Rintala et al. 1997). The chronic feeding models produce damage in the cerebellum, hippocampus, as well as peripheral neuropathy (Cohen et al. 2007; Mellion et al. 2013; Walker et al. 1980). Intragastric gavage models shows similar damage but can be done over the course of a few days in rats or about a week in mice making them less time intensive than the chronic feeding model (Collins et al. 1996; Crews 2008; Hayes et al. 2013; Kelso et al. 2011; Qin and Crews 2012a; Qin and Crews 2012b).

These experiments use a modified version of the Majchrowicz model which exposes rats to alcohol over a four-day period (Majchrowicz 1975). The Majchrowicz model has been chosen to as it mimics the multiple days of binge drinking seen in human alcoholics (Faingold 2008; Tomsovic 1974). It also produces BECs comparable to a subset of alcoholics with higher tolerance that are functional at BECs well above 300mg/dL due to years of alcohol (Cartlidge and Redmond 1990; Urso et al. 1981). Chronic feeding models have lower BECs that may not reflect the alcohol concentrations seen in tolerant alcoholics (Cohen et al. 2007; Mellion et al. 2013). Furthermore, this model produces other traits characteristic of AUDs including tolerance and withdrawal (Table 1.1; Crews and Nixon 2009; Crews 2008). Because tolerance varies among individuals, the Majchrowicz model mimics the human condition and tailors the dose based on behavior unlike other models of alcohol-induced neurodegeneration (Majchrowicz 1975; Penland et al. 2001). Specifics about the procedures of the

Majchrowicz model will be described in the methods section of chapter two, but importantly, it is used here because it produces characteristics traits of AUDs, is a model of binge drinking, and causes neurodegeneration (Crews and Nixon 2009; Crews 2008). Because all of these aspects of ethanol consumption contribute to AUD progression, the Majchrowicz model represents a valid paradigm for understanding alcohol abuse.

Alcohol Use Disorders: Mechanisms of Alcohol-Induced Neurodegeneration

Animal models of alcohol-induced neurodegeneration in conjunction with studies of human alcoholics have increased the understanding of alcoholic brain damage and led to four general proposed mechanisms of neuronal loss: glutamate excitotoxicity, reduced neurogenesis, oxidative stress, and neuroinflammation. Glutamate excitotoxicity involves excess levels of glutamate or increased sensitivity of glutamate receptors that leads to excessive Ca^{2+} influx, neuronal dysfunction and neurodegeneration (Ankarcrona et al. 1995; Lau and Tymianski 2010). During intoxication, ethanol acts as an NMDA antagonist, but as previously stated, chronic ethanol exposure causes an upregulation and supersensitivity of NMDA receptors (Chandler et al. 1993a; Chandler et al. 1993b; Hoffman 1995). *In vivo* studies also suggest that the upregulation and supersensitivity of NMDA receptor results in excess glutamate in the system during withdrawal (Dahchour and De Witte 2003; Grant et al. 1990). Moreover, NMDA receptor antagonists like acamprosate and MK-801 reduce ethanol withdrawal-induced glutamatergic spikes and neurotoxicity (Dahchour et al. 1998; De Witte et al. 2005; Mayer et al. 2002; Prendergast et al. 2004). Together, these studies suggest that glutamate excitotoxicity is a factor in alcohol-induced neurodegeneration, perhaps specifically due to ethanol withdrawal.

A second proposed mechanism of alcohol-induced neurodegeneration is decreased adult neurogenesis (Nixon and Crews 2002). Unlike the glutamate excitotoxicity role of degeneration which focuses on cell death from alcohol withdrawal, alcohol-induced decreases in neurogenesis are seen during intoxication in the absence of withdrawal (Crews and Nixon 2009; Nixon 2006). In the mammalian postnatal brain, neurogenesis constitutively occurs in the hippocampal subgranular zone of the dentate gyrus (DG) as well as in the subventricular zone of the lateral ventricles (Altman and Das 1965; Doetsch et al. 1999; Eriksson et al. 1998). Neurogenesis is a process that can be divided into four components: proliferation, differentiation, migration/integration, and survival (Gage 2000). Reductions in neural progenitor cell proliferation and the long-term survival of newborn cells have specifically been observed in the Majchrowicz model used within these experiments, and studies using vapor exposure concur (Morris et al. 2010a; Nixon and Crews 2002; Richardson et al. 2009). Reduced cell proliferation and neuronal cell survival have both been seen in other neurodegenerative diseases with cognitive deficits (Marxreiter et al. 2013; Ransome et al. 2012) and provide another mechanism by which alcohol abuse could lead to neuronal cell loss (Nixon 2006; Nixon and Morris 2008).

Oxidative stress, specifically an increase in reactive oxygen species (ROS) and the depletion of antioxidant defenses, is associated with neuronal cell death in a variety of neurodegenerative diseases including Alzheimer's and Parkinson's disease (Reynolds et al. 2007). ROS production can cause mitochondrial dysfunction and lead to cellular loss (O'Rourke et al. 2005). Postmortem studies of alcoholic brains indicate there are increases in enzymes associated with ROS production (Qin and Crews 2012b). Models of alcoholic brain damage including the Majchrowicz model concur with findings in alcoholics showing increases in nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase and cyclooxygenase-2 (COX-2) which resulted in tissue damage by producing free radicals (Knapp and Crews 1999; Qin and Crews 2012a; Qin and Crews 2012b; Reynolds et al. 2007). Alternatively, alcohol-induced oxidative stress can be due to ROS produced directly by the metabolism of ethanol at high BECs. Cytochrome P450 2E1 (CYP2E1) preferentially metabolizes alcohol within certain brain regions causing an increase in ROS production (Haorah et al. 2005; Haorah et al. 2008; Ronis et al. 1993). Furthermore, chronic ethanol exposure induces CYP2E1 protein levels, mRNA expression, and activity (Heit et al. 2013; Zhong et al. 2012). Increased ROS production further promotes an environment of oxidative stress by causing mitochondrial dysfunction (Nixon et al. 2009; Reddy et al. 2013).

Although glutamate excitotoxicity disrupted neurogenesis, and oxidative stress were discussed as separate causative factors of degeneration, in reality they can contribute to each other's pathological pathways and likely act in conjunction to lead to neurodegeneration pathways. The final proposed mechanism of ethanol-induced brain damage, neuroinflammation, will be discussed in more detail as it is a focus of this series of experiments. Moreover, the influence of the neuroimmune system, specifically microglia, within each of the other proposed mechanisms of neurodegeneration will also be discussed.

Neuroinflammation

Inflammation is the biological response to noxious stimuli such as invading pathogens, foreign chemicals, or cellular damage. In the periphery, the inflammatory response is characterized by five basic components caused by intracellular immune signaling events (feelings of pain, flushing, swelling, heat, and a subsequent functional deficit; Graeber et al. 2011). Inflammation is the result of an immune response which includes both innate and adaptive immunity. It was originally hypothesized that the

blood brain barrier (BBB) made the CNS an immune impervious system because of the lack of rejection of xenografts by rat brain (Murphy and Sturm 1923). However, the discovery of the innate immune cell, microglia, and their phagocytic capacity within the CNS completely changed this view (Neuwelt and Clark 1978; Penfield 1925). As the innate immune cell, microglial activation alone is often referred to as neuroinflammation, but the heterogeneous nature of microglia makes equating neuroinflammation solely to microglial activation problematic (Carson et al. 2007; Carson et al. 2006). A more encompassing, appropriate definition of neuroinflammation incorporates a complex system consisting of three distinct processes: disruption of the blood-brain barrier, infiltration of T and B lymphocytes, and activation of microglia/macrophages (Carson et al. 2006; Hickey 2001).

The initial discovery of the BBB came in 1885 when it was observed that the injection of dye into the circulatory system did not result in staining of brain tissue (de Vries et al. 1997). Since this initial observation, the actual composition of the BBB has been elucidated. The BBB is a complex system of endothelial cells, astrocytic end feet, perivascular macrophages, and the basal lamina that acts as a barrier separating circulating blood from the brain (Pachter et al. 2003). Disruption of this protecting cellular network is a key component of neuroinflammation as the BBB acts to separate the CNS from various immunomodulators (Hickey 2001). The BBB can be disrupted by mechanical injury such as in traumatic brain injury (Readnower et al. 2010) or by chemical agents that alter the integrity of cells or transporters (Haorah et al. 2007a; Haorah et al. 2005).

When the BBB is disrupted, small lymphocytes from the peripheral system can then enter the parenchyma (Fritz et al. 2000). This infiltration initiates the adaptive immune response involving two basic types of lymphocytes: T cells and B cells. T helper cells,

cluster of differentiation (CD) 4^+, recognize major histocompatibility complex (MHC) class II found on antigen-presenting, activated microglia (Gutcher and Becher 2007; Wraith and Nicholson 2012; Xu and Ling 1994). Upon presentation of antigens by MHC-II molecules, T helper cells become activated and begin to proliferate and secrete autocrines attracting cytotoxic T cells (CD 8^+; Wraith and Nicholson 2012). Cytotoxic T cells bind to MHC-I on the damaged cell and secrete various cytotoxins including perforins and granulysin that leads to cell death (Whitmire 2011). B-cells also mobilize in response to pathogens and bind to T helper cells because of the MHC-II present on B cells (Montecino-Rodriguez and Dorshkind 2006). These cells form an interface known as the immunological synapse that connects the adaptive immune B and T cells with innate immune antigen-presenting cells like activated microglia/macrophages (Davis et al. 1999). The cells within this synapse work in concert with one another to elicit a true neuroinflammatory event and lead to cell death through downstream cell signaling pathways (Chakraborty et al. 2010). Although various cells participate in both the innate and adaptive immune system, the focus of this work is on microglia; therefore microglia and their role in neuroinflammation and within the neuroimmune system will be discussed in greater detail.

Microglia

Microglia are a type of glia or non-neuronal cell within the CNS. The term microglia literally means “small glue”: “small” as in the relative size of microglia compared with other glial cells and “glue” because glial cells were originally thought to hold neurons together (Dermietzel and Spray 1998). However, microglia play a more dynamic role in neuronal homeostasis than simply gluing neurons together (Allen and Barres 2009). Microglia differ from other glial cells such as oligodendrocytes and astrocytes in their origin, morphology and function. Microglia are derived from

hematopoietic cells from precursors that eventually have a macrophage fate rather than neuronal precursor cells (Saijo and Glass 2012; Vilhardt 2005). The origin of microglia makes them uniquely suited to act as an indicator of neuroinflammatory activity. The term indicator was chosen because, as stated earlier, microglial activation alone is not synonymous with neuroinflammation (Hickey 2001). Moreover, both the morphology and function of microglia are diverse, which will be discussed subsequently.

Microglia: Pro versus Anti-Inflammatory State

Microglia become activated in response to various stimuli including neuronal damage, noxious agents, astrocytic secretion, and even more minute neuronal environmental cues like alterations in ion concentrations; however the microglial activation varies or is heterogeneous based on the intensity or type of damage as well as the duration of the insult (Harting et al. 2008; Lai and Todd 2008). Microglia display heterogeneity in their morphology, cytokine secretions, and cell surface proteins (Carson et al. 2007). Distinctions in these attributes are used to categorize microglia in an attempt to understand their function within the CNS under pathological conditions. Although the names used within each classification system are different, the basic premise of all of the classification systems is that microglia are either proinflammatory or anti-inflammatory. For example, Heuschling and colleagues use the terms M1 and M2 to differentiate between pro and anti-inflammatory microglia (Mantovani et al. 2002; Michelucci et al. 2009); whereas, others have simply used the terms classical or partial/alternative activation to describe the heterogeneity of microglial activation (De Simone et al. 2004; McClain et al. 2011). In chapter two, these terms will be broken down even further to reflect the continuum of phenotypes within the proinflammatory and anti-inflammatory states (Raivich et al. 1999a; Raivich et al. 1999b).

In normal, non-pathologic conditions, microglia are in a quiescent state often referred to as “resting.” The term “resting” microglia is a misnomer as quiescent microglia are not actually without function. They are constantly surveying their environment, responding to minute changes within the neuronal milieu (Nimmerjahn et al. 2005). Quiescent microglia have ramified branches from their cell bodies that are used to survey their environment (Fishman and Savitt 1989; Raivich et al. 1999a), but activation by noxious stimuli (i.e. cellular damage, ROS, etc.) alters the morphology of microglia (Brown and Neher 2010; Kettenmann et al. 2011). Resting ramified microglia transform to a “bushy” morphology (Figure 1.1). This bushy shape is characterized by the branches/projections thickening and retracting as well as an enlargement of the cell body (Abraham and Lazar 2000; Morioka et al. 1991; Nimmerjahn et al. 2005). Bushy shaped microglia are often called the partially activated or M2 microglia (Karperien et al. 2013; Raivich et al. 1999a). Upon further or more intense perturbation, the cell becomes rounded in shape as it loses thickened processes and pseudopodia used for motility. Amoeboid microglia are the “classically” defined stage of activated microglia (Figure 1.1; Raivich et al. 1999a).

Figure 1.1 Morphological Diversity of Microglia

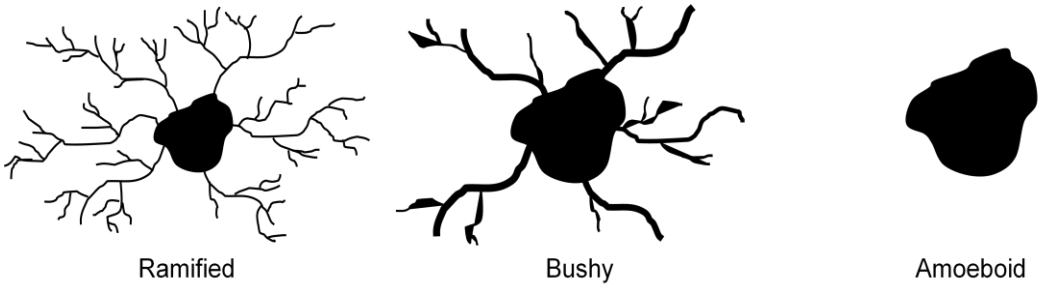

Figure 1.1 Depictions of morphological heterogeneity within microglia activation continuum (adapted from Nimmerjahn et al. 2005).

Because microglial activation is truly a continuum of states, a change in microglial shape alone is not enough to determine whether a cell is pro-versus anti-inflammatory. However, changes in the proteins expressed within the cell accompany the morphological metamorphosis. These alterations in protein expression reflect a change in the function of the microglia. For example, complement receptor 3 (CR3) is an integrin present in all microglia, but its expression is upregulated as a result of chemokines secreted by damaged cells (Akiyama and McGeer 1990; Newton and Hogg 1998). Increased CR3 expression helps microglial cells adhere and anchor to damaged cells as a step in the phagocytic process (Akiyama and McGeer 1990; Hynes 1992; Newton and Hogg 1998). Moreover, phagocytosis of damaged cells also alters microglia protein expression. When microglia internalize or engulf damaged cells, internal proteases (e.g. cathespin S and L) degrade the damaged cell's proteins into MHC-II and the complex is expressed on the cell surface of microglia (Gresser et al. 2001; Nakanishi 2003). Antigen-presenting microglia are a key component of neuroinflammation and the immune synapse as discussed previously. Expression of MHC-II changes the classification of the microglia to a more proinflammatory state (Nakanishi 2003; Xu and Ling 1994) .

Changes in microglia morphology and proteins expressed results in corresponding alterations in secreted cytokines and growth factors that further reflect the function of the cell within the neuronal environment as pro- or anti-inflammatory. For instance, partially activated microglia secrete the anti-inflammatory cytokine interleukin-10 (IL-10) which can suppress other neuroinflammatory factors (Braat et al. 2006; Michelucci et al. 2009). IL-10 suppresses the production of proinflammatory factors by preventing the activation of nuclear factor kappa-light chain enhancer of activated B cells (NF-κB; Correa et al. 2010; Heyen et al. 2000). NF-κB is a transcription factor that is

both activated by and induces the neuroimmune response in a canonical pathway (Kaltschmidt et al. 2005; Vallabhapurapu and Karin 2009). On the other end of the spectrum, when microglia become fully or classically activated, they secrete proinflammatory cytokines like tumor necrosis factor-alpha (TNF-α). Unlike IL-10, TNF-α increases the production of NF-κB as well as members of the caspase family which elicits cascades that promote an inflammatory environment (Gaur and Aggarwal 2003). Altogether, morphological differences coupled with changes in proteins expressed and cytokines secreted can be used to assess the function and role of microglia under pathological conditions. Markers used within this dissertation to assess the state of microglia are presented in figure 1.2.

Figure 1.2 Pro- and Anti-inflammatory Microglial Markers

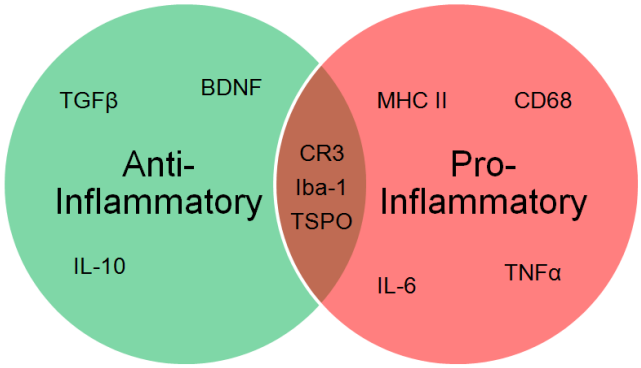

Figure 1.2 Selected markers used within to characterize microglia. Those markers that do not directly indicate pro- or anti-inflammation are placed in the middle.

Microglia: Acute versus Chronic Activation

Although proinflammatory microglia are generally thought to be associated with neuroinflammatory-induced neurodegeneration, the timing of activation and the duration of activation also plays a major role in whether microglia contribute to neurodegeneration. Proinflammatory microglial activation does not always result in

excess damage but can also be associated with recovery. An early, immediate activation of microglia is necessary for recovery. For example, acute microglial activation has been described as participating in “housekeeping” (Nimmerjahn et al. 2005) and “nursing” (Streit 2002b) in the CNS. Activated microglia migrate to damaged areas, and depending on the level of activation, they begin to secrete neurotrophic factors (nurse) or remove debris (housekeep; Petersen and Dailey 2004; Takayama and Ueda 2005). This migration is triggered by chemokines released by damaged neurons and by macrophage colony stimulating factor (MCSF) released by other microglia (Davalos et al. 2005; Gao and Ji 2010; Raivich et al. 1991). MCSF can also promote the proliferation of microglia in response to damage (Carrier et al. 2004; Kloss et al. 1997). This response increases the microglia in the area that are supporting damaged cells and removing neurons beyond repair (Carson et al. 2007).

However, the chronic activation of microglia is associated with neuronal loss and has been proposed as a mechanism within various neurodegenerative diseases (Amor et al. 2010). For example, in traumatic brain injury, microglial activation persists well after the initial focal brain injury and causes secondary damage outside of the original mechanical injury (Lenzlinger et al. 2001; Ramlackhansingh et al. 2011). While differences in the type of activation can affect the contributions of microglia to neurodegeneration, the timing and duration of microglial activation is just as important to understand whether these pro- and anti-inflammatory roles are indicative of neurodegeneration or are participating in recovery from damage.

Microglia: Glutamate Excitotoxicity, Oxidative Stress, & Neurogenesis

Glutamate Excitotoxicity

Not only are microglia indicators of potential neuroinflammation, but microglia also have roles in the other proposed mechanisms of ethanol brain damage: glutamate excitotoxicity, oxidative stress, and reduced neurogenesis. The excessive glutamate levels that mediate glutamate excitotoxicity occur because of both increased release as well as decreased uptake. Microglia have the capacity to affect both processes that control glutamate excitotoxicity. For example, TNF-α secreted by activated microglia can initiate the release of glutamate from microglia cells (Takeuchi et al. 2006; Yin et al. 2012). Microglial release of glutamate could contribute to glutamate excitotoxic alcohol-induced neurodegeneration. However, activated microglial cells also upregulate their expression of the glutamate transporter 1 (GLT-1; Persson et al. 2005; van Landeghem et al. 2001). GLT-1 uptakes glutamate into the microglial cell where it can be recycled by glutamine synthetase (Aschner 2000; Chretien et al. 2002). Glutamate uptake and degradation by glia would be neuroprotective by reducing the levels of glutamate in the synapse (Gras et al. 2003).

Oxidative Stress

Microglia are both sources of ROS and are activated by increased ROS production. CR3, previously discussed for its role in phagocytosis, has been shown to be upregulated by ROS indicating that microglia activation is sensitive to oxidative stress (Roy et al. 2008). Activated microglia can then be a source of ROS by releasing superoxide, hydrogen peroxide, hydroxyl free radicals from NADPH oxidase phagocytic reactions (Block et al. 2007; Reynolds et al. 2007). This release of ROS, like so many other facets of microglia activation, is also directed by proinflammatory cytokines like TNF-α (Smith et al. 2012). The role of microglia within oxidative stress further implicates microglia activation as a potential source of neurodegeneration.

Neurogenesis

The heterogeneity of microglial activation is reflected in its effects on neurogenesis also (Kohman and Rhodes 2013; Morrens et al. 2012). The balance of microglia-derived cytokines and growth factors can regulate of adult hippocampal neurogenesis (Ekdahl et al. 2009). Specifically, the type of cytokines secreted by microglia affects neurogenesis (Butovsky et al. 2006; Ekdahl et al. 2009). When fully or classically activated, microglia secrete proinflammatory cytokines that are generally associated with reductions in normal adult neurogenesis (Ekdahl et al. 2003; Monje et al. 2003). These reductions in neurogenesis can occur due to various effects on neurogenesis. For example when interleukin 6 (IL-6), a proinflammatory cytokine, is produced, it results in decreased proliferation (Vallieres et al. 2002); whereas other proinflammatory cytokines such as interferon gamma (IFN-γ) can dysregulate differentiation, changing the fate of newborn cells from neuronal to astrocytic (Walter et al. 2011; Yong et al. 1991). On the other end of the continuum, microglial activation is necessary for reactive neurogenesis in response to neuronal damage (Deboy et al. 2006; Wainwright et al. 2009). In an adrenalectomy model of reactive neurogenesis, blocking transforming growth factor-beta (TGF-β) receptors reduced neurogenesis (Battista et al. 2006) whereas increases in IL-10 enhanced neurogenesis (Kiyota et al. 2012).

As previously described, microglia have the propensity to affect various mechanisms of alcohol-induced neurodegeneration as well as recovery. Figure 1.3 depicts the ways in which microglia could be involved in recovery mechanisms. The complex nature of microglia makes understanding the characteristics of microglia following ethanol exposure of distinct interest. This dissertation focuses on the pro- or anti-inflammatory state of microglia as well as the initiation and duration of activation as an indicator of its role in alcohol-induced damage and/or recovery.

Figure 1.3 Potential roles of microglia in alcohol-induced damage and recovery

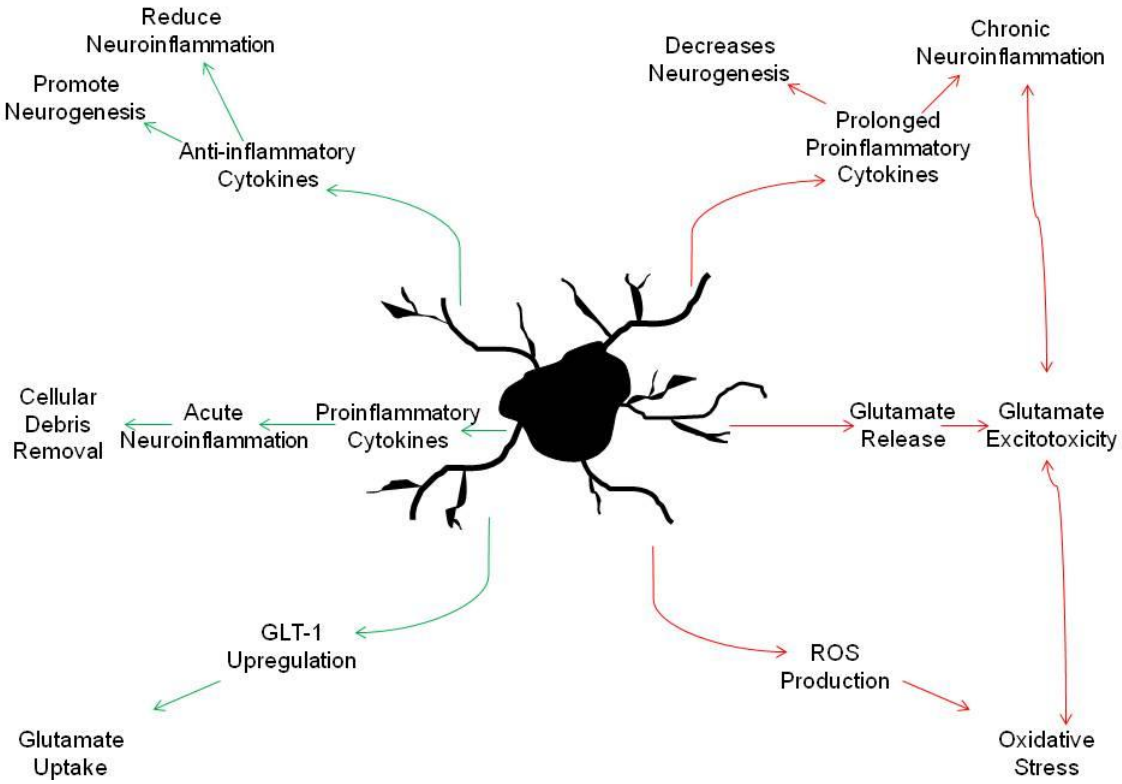

Figure 1.3 Examples of the duality of microglia in promoting recovery mechanisms (green arrows) and contributing to neuronal damage (red arrows)

Alcohol & Neuroimmune System

Alcohol modulates the immune system of various organ systems including but not limited to the respiratory, musculoskeletal, and digestive system. Whether alcohol is an immunosuppressant or immunoactivating agent varies within each system (Molina et al. 2010). The digestive system, specifically the liver has been a major focus of studies examining the effects of alcohol on inflammation and the immune system. This focus is mainly due to the common occurrence of liver cirrhosis in alcoholics (Beier and McClain 2010; Wang et al. 2012b). Studies looking at alcoholic liver cirrhosis have shown the effects of alcohol on monocytes in the periphery. Monocytes isolated from the blood of

alcoholics have greater basal expression of proinflammatory cytokines as well as react more robustly to challenges with lipopolysaccharide (LPS; Barve et al. 2006; McClain and Cohen 1989). The fact that microglia are the monocytes of the CNS suggests that microglia likely would also be affected by chronic ethanol exposure.

Initially few studies examined microglia as it was originally proposed that alcohol-induced brain damage was too low and chronic to perturb microglia (Streit 1994). However, recent trends have shown a marked increase in the literature exploring the neuroimmune system in alcohol and drug abuse (Coller and Hutchinson 2012; Cui et al. 2011). The brains of human alcoholics have shown some indices of microglial activation. Increases in the microglial secreted protein monocyte chemoattractant protein (MCP-1) were seen in various regions of the mesolimbic pathway including the ventral tegmental area, the substantia nigra, the amygdala, and importantly for this work, the hippocampus (He and Crews 2008). As the name implies, MCP-1 is a chemokine that causes the congregation of monocytes and T cells by initializing the motility of microglia/ macrophages (Carr et al. 1994; Hinojosa et al. 2011). Accompanying the increase in the MCP-1 were increases in markers of microglia activation (He and Crews 2008). However, neither microglial activation nor attraction by MCP-1 within an area is enough to indicate a proinflammatory state nor causation between microglia activation and AUD associated neurodegeneration (Hickey 2001; Hinojosa et al. 2011). These results however do imply that chronic ethanol exposure affects the neuroimmune system.

Studies of postmortem brains of alcoholic agree with studies looking at microglial activation that the neuroimmune system is altered within AUDs, but chronic alcohol consumption causes dysregulation of the NF-κB system (Okvist et al. 2007). Chronic ethanol exposure down regulated mRNA levels associated with the innate immune system as well as decreased NF-κB binding to DNA within the prefrontal cortex (Liu et

al. 2006; Okvist et al. 2007). While these studies done on the brains of postmortem alcoholics do not agree on the direction of the effects of alcohol on potential neuroinflammatory signaling, together, they indicate that chronic alcohol exposure results in neuroadaptations that alter the normal neuroimmune function.

To truly appreciate alcohol's modulatory effects on the neuroimmune system requires AUD models. The vast majority of the work looking at alcohol's influence on the neuroimmune system has been done *in vitro* or in rodent models of AUDs examining the effects of alcohol on immune gene responses, BBB disruption, astrocytic activation, and finally microglial modulation. *In vitro* studies using organotypic hippocampal-entorhinal cortex cultured brain slices and animal models have confirmed results seen in human alcoholics showing modulation of the NF-κB system (Crews et al. 2006a; Zou and Crews 2010). However, these studies only show an upregulation of NF-κB as well as increased binding (Crews et al. 2011; Zou and Crews 2010). This effect differs from observations in the brains of human alcoholics where genes within the NF-κB were both up and down regulated (Okvist et al. 2007; Zou and Crews 2010). The differences measured are not surprising given the transient nature of many responses in the immune system including NF-κB upregulation (Cechetto 2001). Many *in vitro* studies look at the effects of alcohol on the neuroimmune system during ethanol exposure. However, alcohol abuse is driven by phasic patterns of use including periods of intoxication, acute withdrawal, and abstinence (Heilig et al. 2010). Studying the effects of the ethanol on the neuroimmune system during these different periods gives a fuller view of how neuroinflammation may be involved with damage. Moreover, AUD models, including the Majchrowicz model, indicate that neurodegeneration can occur during intoxication and in abstinence making studying neuroimmune changes in a timeline crucial.

Disruption of the BBB is a major component of a neuroinflammatory response (Hickey 2001). The increase in MCP-1 seen in the brains of post-mortem human alcoholics is of interest not only due to its role in attracting glial cells but also because MCP-1 is associated with the breakdown of the BBB (Stamatovic et al. 2003; Stamatovic et al. 2005). However, despite increases in MCP-1, no direct evidence of BBB disruption within human alcoholics exists. *In vitro* models using human epithelial cells to mimic the BBB have found damage to the cells indicative of BBB disruption. In these models, ethanol disrupts proteins associated with tight junctions as well as indirectly causes endothelial cell through ROS production both of which can lead to BBB disruption (Haorah et al. 2007a; Haorah et al. 2005; Haorah et al. 2007b). The integrity of the BBB is vital to controlling inflammatory events and compromising it is just one more possible cause of damage (Russo et al. 2011). The integrity of the BBB will be examined in experiments presented in chapter two.

In vitro and *in vivo* models of AUD studies have consistently shown changes in glial cells and their function in response to ethanol. Although the focus of this dissertation is the effects of ethanol on microglia, astrocytes play a major role in neuroimmune function and therefore cannot be ignored (Dong and Benveniste 2001). Both *in vitro* and *in vivo* models have shown that astrocytes are affected by ethanol exposure but results differ based on whether ethanol is present in the culture or animal, respectively (DeVito et al. 2000; Franke et al. 1997; Kane et al. 1996; Kelso et al. 2011). For example, during abstinence glial fibrillary acidic protein (GFAP), an immunohistochemical marker of astrocytes, is upregulated indicating that astrocytes are activated in recovery from ethanol (Hayes et al. 2013; Kelso et al. 2011), but *in vitro* studies suggest that ethanol would inhibit the proliferation of astrocytes during intoxication (Kane et al. 1996). Furthermore, ethanol's effects on astrocytes have been

implicated in other problems associated with chronic ethanol use that would affect the neuroimmune system and/or mechanisms of neurodegeneration including disruptions of the BBB (Abdul Muneer et al. 2011), glutamate excitotoxicity (Miguel-Hidalgo 2006; Wu et al. 2011), and ROS production (Gonthier et al. 1997; Jin et al. 2013). The astrocytic contribution to the neuroimmune reaction is important, but these studies focused on microglia.

Alcohol & Neuroimmune System: Microglia Activation

Much like the effects of ethanol on astrocytes, various models of alcohol abuse agree that microglia are activated following ethanol exposure (Kelso et al. 2011; McClain et al. 2011; Ward et al. 2009a; Zhao et al. 2013), but the phenotype, initiation, and duration of microglia activation within these models is not as clear. Some have discussed microglial activation as initiating a neuroinflammatory response that leads to neurodegeneration (Crews et al. 2011; He et al. 2005; Qin and Crews 2012a; Qin and Crews 2012b; Qin et al. 2008). The majority of the "neuroinflammation driving AUD neurodegeneration" studies looks at the neuroimmune response during intoxication and do not consider immune response as a necessary function to restore homeostasis. The duration and timing of microglial activation is just as important as the type of activation. For example, the Crews lab has indicated that increases in TNF-α concentrations following ethanol exposure maybe a causative factor in neurodegeneration (Crews et al. 2006b; Qin et al. 2008), but acute increases in proinflammatory cytokines can actually promote neuroprotection (Song et al. 2013; Turrin and Rivest 2006). Furthermore, studies in other models of alcohol-induced neurodegeneration have not observed proinflammatory cytokines either during intoxication or in abstinence and suggest that microglial activation may be involved with recovery (McClain et al. 2011; Zahr et al. 2010a). Similar to controversies regarding cytokines induced by ethanol exposure,

some studies have described more classical signs activation of microglia looking at the proteins expressed such as increases in MHC-II (Ward et al. 2009a) and phagocytic activity (Zhao et al. 2013), while others have only seen evidence of low grade partial activation (McClain et al. 2011; Nixon et al. 2008).

The level of activation is not the only point of contingency regarding ethanol's effect on microglia as the source of activation is not clear. Studies looking at astrocytic and microglial cultures suggest that the toll like receptor 4 (TLR4) cell signaling cascade can be directly induced by ethanol (Blanco et al. 2005; Fernandez-Lizarbe et al. 2013; Fernandez-Lizarbe et al. 2009). It has been proposed that ethanol modulates TLR4 signaling by modulating lipids within the cell membrane of glial cells (Blanco et al. 2008; Fernandez-Lizarbe et al. 2013; Fernandez-Lizarbe et al. 2008). Direct ethanol induction of TLR4 signaling would suggest that microglia activation is the result of an inflammatory response of astrocytes. However, others using in vivo models suggest that microglial and astrocytic activation occurs as a result of neuronal damage and is subsequent to neurodegeneration (Kelso et al. 2011; McClain et al. 2011). The chronological order of events indirectly implies causation and is a crucial aspect of understanding the role of microglial activation within AUDs. If microglia are activated prior to neurodegeneration, it implies that the neuroimmune response may mediate neuronal damage. However, if microglia activation is a consequence of damaged cells, the neuroimmune response may initiate as a recovery mechanism. Both the type of microglial activation and the chronology of evidence of activation and degeneration will be determined in experiments presented in chapters two and three.

Alcohol & Neuroimmune System: Microglial Priming

One of the key contributing factors of the theory that chronic alcohol consumption causes neurodegeneration through a microglial associated neuroinflammatory response

is that the microglial response is exacerbated or perpetuated by influences from the systemic system (Crews 2012; Cunningham 2013; de la Monte et al. 2009). The basic premise of this theory is that chronic ethanol exposure disrupts the BBB and allows the infiltration of peripheral immunomodulators such as activated peripheral macrophages and their associated cytokines that then alter microglia activation and the neuroimmune signaling (Crews 2012; Crews et al. 2011). Studies have shown that persistent activation of microglia following an initial damaging event can impact the neuroimmune system by modulating secondary or future microglial responses to other immune challenges (Dilger and Johnson 2008; Norden and Godbout 2013). This phenomenon has been referred to as microglial priming and has been shown to affect the neuroimmune response for extended periods. Primed microglia exhibit a more robust proinflammatory response upon secondary activation. For instance, early-life infection in rodents caused microglial to be activated (Bilbo and Schwarz 2009). This microglial activation persisted into adulthood to a lower degree than the initial adolescent response; however, upon subsequent immunological challenge, primed microglia produced higher levels of proinflammatory cytokines compared with microglia from rodents without an early life infection. This exacerbated response months after the initial damaging event resulted in deficits in neurogenesis as well as cognition (Bilbo and Schwarz 2009; Bland et al. 2010; Williamson et al. 2011). In support of this phenomenon in AUDs, studies show that prior ethanol exposure exacerbates the microglial response to LPS and polyinosinic:polycytidylic acid (Poly IC; Qin and Crews 2012a; Qin et al. 2008). Both LPS and Poly IC at the doses used produce a robust immune response, which complicates the interpretation of these studies (Qin and Crews 2012a; Qin et al. 2008). In chapter four, the ability of ethanol alone to act as a “secondary hit” to a primed response will be considered. Determining if ethanol alone exacerbates microglia activation is important as

alcohol-induced neurodegeneration is seen independent of liver cirrhosis, the hypothesized source of systemic inflammation (Harper and Matsumoto 2005; Zahr et al. 2009).

Project Overview

Neuroplastic changes that occur from chronic alcohol consumption are one potential underlying event in the progression of an AUD. One such neuronal consequence is neurodegeneration in the corticolimbic pathway. Understanding the mechanisms that lead to neuronal damage may partially shed light on the progression of AUD development. The current dissertation examines neuroinflammation as a potential mechanism of alcohol-induced neurodegeneration, specifically by investigating the effects of ethanol on microglia. A rat model of an AUD known to cause neurodegeneration was used to determine the phenotype and persistence of microglia activation from varying durations of ethanol exposure. The overarching hypothesis for this project is that **alcohol exposure elicits a differential response on microglia depending on the duration of ethanol exposure as well as whether activation is measured during intoxication or abstinence.**

Aim 1: Determine the phenotype of microglia reactivity following binge ethanol exposure (Chapter 2).

We hypothesize that binge ethanol exposure induces low-grade, partial microglia activation. The phenotype of activation will be determined following ethanol exposure examining proteins expressed within microglia using autoradiography and immunohistochemistry to examine. The microglial phenotype will also be assessed using ELISAs to look at cytokine expression.

Aim 2. Determine the earliest indices of microglial activation in the Majchrowicz model of an AUD (Chapter 3).

We hypothesize that the initial microglial response will occur subsequent to days of ethanol exposure previously shown to cause neurodegeneration. *[^{3}H]-PK-11195,* a

sensitive microglial activation marker, will be used to determine the earliest indices of microglial activation, and microglial cell counts will be used to ensure that measurements of *[^{3}H]-PK-11195 are based on activation and not changes in cell number*.

Aim 3. Determine if alcohol-induced microglia reactivity following the Majchrowicz model is “primed” (Chapter 4).

We hypothesize that a second binge ethanol exposure will potentiate the microglia response seen after binge ethanol exposure and produce classical signs of activation. Microglial activation phenotype will be determined following a second ethanol exposure using immunohistochemistry to look at the markers indicative of pro- versus anti-inflammation, whereas ELISAs will be used to assess function by looking at cytokine expression.

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Chapter 2: Microglial activation is not equivalent to neuroinflammation in alcohol-induced neurodegeneration: the importance of microglia phenotype

INTRODUCTION

Whether microglial activation is the cause or consequence of neurodegeneration is a hotly debated topic in studies of neurodegenerative disease. Although not traditionally classified as a neurodegenerative disease due to its preventable nature, AUDs and specifically the characteristic excessive consumption of alcohol, result in corticolimbic neurodegeneration that underlies a variety of cognitive deficits in alcoholics (Crews and Nixon 2009; Obernier et al. 2002a; Pfefferbaum et al. 1992; Sullivan et al. 1995). As alcohol-induced neurodegeneration is thought to be a critical step in the development of an AUD (Crews and Boettiger 2009; Crews et al. 1999; Koob and Le Moal 1997), understanding how excessive alcohol consumption results in neuronal loss is crucial for the development of prevention and treatment strategies. It has been hypothesized that alcohol-induced neuroinflammation directly contributes to neurodegeneration and the development of AUDs (Crews et al. 2011). Neuroinflammation has been inferred from the upregulation of a variety of proinflammatory genes and cytokines involved in the innate immune system (Crews et al. 2006b; He and Crews 2008; Knapp and Crews 1999; Qin et al. 2008). For example, chronic ethanol exposure induces innate immune signaling cascades through activation of the proinflammatory transcription factor, NF-κB (Crews et al. 2006b; Crews et al.

2011; Valles et al. 2004). Others have shown that a variety of proinflammatory signals are associated with increased ethanol drinking and preference (Blednov et al. 2012) and that peripheral inflammation promotes increases in voluntary ethanol intake whereas anti-inflammatory administration reduces its consumption (Agrawal et al. 2011; Blednov et al. 2011). However, remarkably little is known about the effects of alcohol on microglia, the primary mediators of the innate immune system in the brain.

Microglial activation, the process in which microglia alter their morphology and functionally differentiate in response to changes in their environment, was traditionally described as proinflammatory and cytotoxic (Kreutzberg 1996). In normal, non-pathologic conditions microglia are generally in a quiescent state often referred to as "resting." Quiescent microglia, however, are not truly resting; their highly ramified morphology reflects their constant surveying of the surrounding environment (Fishman and Savitt 1989; Nimmerjahn et al. 2005). For many neurodegenerative disorders, activated microglia are a hallmark of neuroinflammation (Banati et al. 1993; Block and Hong 2005; Colton and Gilbert 1987; Woodroofe et al. 1991). However, more recent work demonstrates that it is not just whether microglia are activated, but more importantly their phenotype during activation (Carson et al. 2007; Colton and Wilcock 2010; Kreutzberg 1996; Raivich et al. 1999b). Various terms have been used to describe a perceived dichotomy in microglial phenotype including M1 versus M2, classical versus alternative and classical versus partial activation. However, all classify microglia into one of two categories when it is a spectrum of phenotypes or behaviors that exist. For example, microglia phenotype varies with the type of insult, the extent of damage, and the time of recovery post injury, which makes it necessary to thoroughly examine phenotypic hallmarks within a disease before inferring their role in neuroinflammation (Harting et al. 2008; Lai and Todd 2008; Saijo and Glass 2012).

Application of the idea of graded levels of activation allows for investigation of a potential spectrum of phenotypes. As such, Raivich defines 5 levels of microglial activation or phenotypes (Table 2.1): resting (stage 0), alert (stage 1), homing (stage 2), phagocytic (stage 3a) and bystander activation (stage 3b), which can be differentiated by both morphology and cytokine and/or growth factor upregulation (Raivich et al. 1999a). For example, amoeboid morphology and expression of proinflammatory factors such as TNF-α, IL-1ß, prostaglandins, superoxides and nitric oxide, characterize the highest level of activation whereas microglia in lower grades of activation release neuroprotective factors such as IL-10, TGF-ß, and neurotrophins and have a more ramified morphology (Block and Hong 2005; Raivich et al. 1999b). Furthermore, although fully activated microglia are one component of classical inflammation, observation of "activated" microglia alone is not equivalent to nor very informative about the inflammation state (Graeber et al. 2011). Therefore, determining the phenotype of microglia in injury is necessary to understand their role as cytotoxic or neuroprotective and whether they are truly neuroinflammatory (Colton and Wilcock 2010; Kreutzberg 1996; Vilhardt 2005).

A role for cytotoxic microglia in alcohol-induced brain damage has been suggested since the 1990s, however direct evidence of alcohol-induced full or classical microglia activation has yet to be described. The lack of classical signs of activation led some to suggest that the damage in alcoholism is "too chronic" (Streit 1994) or too low level to affect microglia (Kalehua et al. 1992); however, there is evidence of some level of activation in both animal models and human postmortem alcoholic brain. For example, early work showed an upregulation in the microglial marker, [^{3}H]-PK-11195, binding months after alcohol exposure in a four-day binge model of alcoholic neurodegeneration (Obernier et al. 2002b). Later, an unexpected discovery of microglial

proliferation was found in this same model (Nixon et al. 2008). More recently, upregulation of various microglial markers have been described in animal models (McClain et al. 2011), and even led some to conclude that excessive alcohol exposure produces “neuroinflammation” (Qin et al. 2008; Ward et al. 2009b). Importantly, although evidence of microglial activation has been observed in human alcoholic brain samples, the phenotype of these alcohol-activated microglia has yet to be described (Crews et al. 2006b; Crews et al. 2011; He and Crews 2008). Unfortunately, the pervasive theme of these and other papers is that the observation of any marker of activation is equivalent to neuroinflammation. The assessment of single markers of activation is not sufficient to characterize the activation phenotype of microglia and as discussed above, not indicative of inflammation (Colton and Wilcock 2010).

Table 2.1 Microglia Heterogeneity

	Microglial Characteristics	Morphology and Markers	Cytokines
Stage 0	Normal-Ramified	Morphology: long ramified processes	
Stage 1	Alert: thicker processes	Less ramified, thicker processes; ⇑OX-42	TGF-ß1
Stage 2	Homing, Proliferation	Bushy; Proliferation markers	IL-10
Stage 3a	Clustered phagocytes	Amoeboid; possible ⇑MHC-I, ED-1 (CD68)	IL-6, TNF-α
Stage 3b	Bystander activation; Lymphocyte binding	⇑MHC-I, Lower ICAM than 3a	IFN-γ

Table 2.1 Microglial activation can be differentiated based on morphology and marker expression (derived from Raivich et al., 1999a). The cytokines denoted are indicative of a change in expression. For example, microglia characterized as 3a will still express IL-10 but in addition will secrete proinflammatory cytokines such as IL-6 at higher concentrations. An immune response can occur independent of activation and may be observed in Stages 1 – 3 as evidenced by increased MHC-II (OX-6).

The current experiments examine how ethanol exposure, in a well-established model of an AUD that includes significant alcohol-induced neurodegeneration, affects

microglia within the context of classical definitions of inflammation. Specifically, inflammation is defined as a "multicellular process characterized by changes in the vasculature and infiltration of mobile cells." (p. 3800; Graeber et al., 2011). This study uses an extensive assessment of immunohistochemical, morphological, and functional indices of microglial activation in order to determine their phenotype in the hippocampus and entorhinal cortex, regions consistently damaged in this binge paradigm (Collins et al. 1996; Obernier et al. 2002a). Alcohol's effect on the integrity of the BBB was also examined, as macrophage and/or lymphocyte infiltration is a defining phenomenon in inflammation (Hickey 2001).

MATERIALS AND METHODS

Alcohol Administration Model

Rats were subjected to a four-day binge model of alcohol exposure modified from Majchrowicz (1975). This model is designed to mimic the high blood alcohol levels of pattern binge drinkers (Hunt 1993; Tomsovic 1974) and was chosen for its well-documented neurodegeneration profile (Crews 1999; Kelso et al. 2011). All procedures performed were in accordance with the University of Kentucky Institutional Animal Care and Use Committee and aligned with the Guidelines for the Care and Use of Laboratory Animals (NRC, 1996). A total of 214 adult male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were used across all experiments. Animals were 275-300g upon arrival and single-housed in a University of Kentucky AALAC accredited vivarium with a 12h light:dark cycle and had *ad libitum* food and water access unless otherwise noted. Rats were allowed to acclimate to the vivarium for five days but were handled for three days before the binge began to reduce anxiety associated with handling.

Rats were divided into two groups of comparable weights and received either ethanol (25% w/v) or control diet (isocaloric amounts of dextrose) in Vanilla Ensure Plus®. Diet was given every 8h for 4 days via intragastric gavage. During the four days of diet administration, chow was removed and returned 8h after the last dose. Initially, each rat received a 5g/kg dose of ethanol with subsequent doses titrated based on intoxication behavior according to a 6-point scale modified from Majchrowicz (1975) but identical to previously published methods (Morris et al., 2010b; Nixon and Crews, 2004). For example, an animal that simply seems ataxic would receive more ethanol than one that who has lost its righting reflex (Table 2.2). Ethanol animals with intoxication scores of four or greater were given 2mL of water to avoid dehydration. Control animals received the average volume given to the ethanol group to control for neuroplastic changes associated with caloric intake (Gillette-Guyonnet and Vellas 2008; Loncarevic-Vasiljkovic et al. 2012).

Table 2.2 Intoxication Scale

Intoxication Score	Behavioral Attributes	Ethanol Dose (g/kg)
0	Normal animal	5
1	Hypoactive, mildly ataxic	4
2	Ataxic, elevated abdomen	3
3	Ataxic, absence of abdominal elevation, delayed righting reflex	2
4	Loss of righting reflex, retain eye blink reflex	1
5	Loss of righting reflex, loss of eye blink reflex	0

Table 2.2 Animals CNS depression (intoxication) was scored based on behavioral attributes to determine the appropriate ethanol dose.

Ninety minutes after the seventh session of ethanol dosing, tail blood samples were collected. This time point represents the peak intoxication profiles from intragastric gavage studies in rats (Kelly et al. 1987; Livy et al. 2003). Samples were centrifuged for 5 min at 1800g to separate plasma from red blood cells and stored at -20˚C to avoid sample degradation. BECs were determined from 5μL of supernatant serum using an AM1 Alcohol Analyser (Analox, London, UK). Each sample was run in triplicates calibrated against a 300mg/dL external standard and the average expressed as mg/dL. The AM1 Alcohol Analyser works by measuring the oxygen consumption in the oxidation of alcohol to acetaldehyde and hydrogen peroxide (Analox 2007).

Ten hours following the last dose of ethanol, withdrawal was observed for 30 minutes every hour for 16 intervals. Withdrawal behaviors were scored based on a scale modified from Majchrowicz (Majchrowicz 1975; Penland et al. 2001) but identical to that reported previously (Table 2.3; Morris et al., 2010b). Because microglia respond quickly to changes in homeostasis (Davalos et al., 2005; Nimmerjahn et al., 2005) but also have the capacity for persisting memory (Bilbo and Schwarz, 2009; Bland et al., 2010; Williamson et al., 2011) this study examines microglial changes immediately following ethanol exposure through 28 days of abstinence. Therefore, rats were euthanized at various timepoints within this range following binge treatment: T0 (e.g. 0 days after the last dose, specifically within hours), T1, T2, T4, T7, and T28.

Table 2.3 Withdrawal Scale

Withdrawal Score	Behavioral Attribute
1.0	Hyperactivity
1.4	Tail Tremor
1.6	Tail Spasm
2.0	Caudal Tremor
2.2	Tip Toe Arch
2.4	Splayed Limbs
2.6	General Tremor
3.0	Head Tremor
3.2	Induced Running
3.4	Wet Dog Shakes
3.6	Chattering teeth
3.8	Spontaneous Convulsions
4.0	Death

Table 2.3 Animals' behavior was scored based on a modified scale of withdrawal symptoms (Majchrowicz, 1975; Penland et al., 2001).

Autoradiography

Autoradiography was conducted as described in previous reports (Kelso et al., 2006; Sparks and Pauly, 1999). Rats were rapidly decapitated and extracted brains were immediately frozen in isopentane and sliced at 16µm with a cryostat. Two controls were euthanized at each time point and pooled into a single control group for comparison with ethanol treated groups (Readnower et al., 2010). Sections were mounted in a 1 in 8 series on glass slides so that every eighth section was used and stored at -80˚C until processing. Slides were thawed and incubated in 50mM Tris HCl (pH=7.4) buffer with 1nM [^{3}H]-PK-11195 (PerkinElmer, Boston, MA) for 2h followed by a series of washes in 50mM Tris HCl. [^{3}H]-PK11195 specifically binds to the mitochondrial translocator protein 18kDa (TSPO), a protein that is highly upregulated in activated microglia and is associated with cholesterol transport (Kelso et al. 2009; Stephenson et al. 1995; Veiga et al. 2007). Similar to other studies of microglial activation after brain insult, autoradiographic localization of TSPO was used in this study because of its high sensitivity to detect activated microglia (Benavides et al. 2001; Readnower et al. 2010).

After drying, the slides were exposed to BioMax film (Kodak, Rochester, NY) for 6 weeks. The film was developed with GBX developer (Kodak) and analyzed using ImageJ (Scion Imaging; Frederick, Maryland) to determine the relative binding levels by optical density. Sections between approximately between Bregma -2.50mm and -4.00mm, which included both the hippocampus and entorhinal cortex, were quantified (Paxinos and Watson, 2009).

Immunohistochemistry

Rats were overdosed with anesthetic (Nembutal® 100mg/kg; ip) and transcardially perfused with 0.1M phosphate buffered saline (PBS, pH=7.4) followed by 4% paraformaldehyde in PBS. Brains were extracted, postfixed in paraformaldehyde for 24 hours (ED-1, OX-6, Iba-1, and IgG) or 1 hour (OX-42), and sectioned coronally at 40µm using a vibrating microtome (Leica VT1000S; Wetzlar, Germany). Sections were collected in a 1:12 series and stored in cryoprotectant at -20˚C until processing so that every twelfth section was stained for each antibody of interest. Free floating tissue was washed in tris buffered saline (TBS, pH=7.5) and endogenous peroxidases quenched with 0.6% H_2O_2 in TBS. Following additional washes, sections were blocked for nonspecific binding (TBS, 0.1% triton X-100, and 3% horse or goat serum), and then incubated overnight in primary antibody at 4˚C as follows: mouse anti-OX-6 (1:500, Serotec, Raleigh, NC), mouse anti-ED-1 (1:500; Serotec), rabbit anti-Iba-1 (1:1000, Wako, Richmond, VA), or mouse anti-OX-42 (1:1000; Serotec)

Primaries were chosen for their specificity for activated microglia phenotypes (Table 2.1). The Iba-1 antibody recognizes a 17kDa EF hand protein that is similar in structure to other calcium binding proteins such as calmodulin (Heizmann and Hunziker 1991; Imai et al. 1996; Ito et al. 1998). Iba-1 is used to mark all microglia, but it is upregulated during activation as it is associated with the release of cytokines, adhesion,

and proliferation (Donato 1999; Donato 2003; Hwang et al. 2006). The OX-42 antibody is also constitutively expressed in all macrophages and recognizes CR3 or CD11b (Robinson et al., 1986). Upregulation of this receptor is one of the first indices of activation as microglia prepare to adhere to damaged cells (Hynes, 1992; Morioka et al., 1992). Unlike Iba-1 and OX-42, ED-1 and OX-6 are not expressed in all microglia. The ED-1 antibody, also known as anti-CD68, recognizes a glycoprotein on the lysosomal membrane of macrophages and microglia that is indicative of phagocytic activity (Bauer et al., 1994; Damoiseaux et al., 1994). ED-1 is typically used to determine the presence of classically or fully activated phagocytic microglia (Graeber and Streit 2009; O'Keefe et al. 2002; Raivich et al. 1999a). The OX-6 antibody recognizes MHC-II associated with induction of T-helper cells (O'Keefe et al., 2002; Raivich et al., 1999a). Although OX-6 is also associated with the recruitment of phagocytes and is considered a hallmark of an immune response (Kaur and Ling 1992; McGeer et al. 1993), recent work suggests that it may also be expressed in partially activated microglia (Colton and Wilcock, 2010). Microglia exhibit weak antigen-presenting capabilities, but many neuroinflammatory reactions involve the upregulation of microglial MHC-II (Zhang et al. 2011).

Methods for the application of secondary antibody (biotinylated horse anti-mouse, rat adsorbed, or biotinylated goat anti-rabbit, Vector Laboratories, Burlingame, CA), avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) and chromagen, nickel-enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences, Warrington, PA), were identical for all primary antibodies and followed previously published methods (McClain et al., 2011).

To determine if infiltration of macrophages and lymphocytes could occur in this model, BBB impairment was examined. Tissue was incubated in biotinylated rabbit anti-rat IgG for 2 hours followed by detection with ABC and the chromagen DAB

(Rabchevsky et al. 1999; Schmidt-Kastner et al. 1993). The IgG antibody is a marker of immunoglobulin G. With an intact BBB, immunoglobulins would remain in the peripheral system due to a lack of transport mechanisms (Triguero et al. 1989); thus, the presence of IgG in the brain parenchyma indicates BBB disruption. Following the final wash, all stained sections were mounted onto glass slides and dried before being coverslipped with Cytoseal® (Stephens Scientific, Wayne, NJ).

Quantification

All sections were coded to ensure the experimenter was blinded to treatment conditions during quantification. All analyses were conducted on an Olympus BX-51 microscope (Olympus, Center Valley, PA), with motorized stage (Prior, Rockland, MA), microcator and DP70 digital camera (Olympus). OX-42 immunoreactivity was analyzed using Visiomorph image analysis program (Visiomorph, Hørsholm, Denmark). Using a 10x objective lens, regions of interest were drawn around the hippocampal subregions and the entorhinal cortex approximately between Bregma -2.50mm and -4.00mm as determined by Paxinos (Paxinos and Watson, 2009). Immunoreactivity was determined by optical density and the percent area of staining was obtained. Images were run in a batch process, and immunoreactivity was calculated and expressed as percent control. Sections in the same stereotaxic regions were assessed qualitatively for the presence of ED-1, OX-6 and IgG using a 10x objective.

Iba-1+ cells were quantified in the entorhinal cortex by an image analysis system. Multi-panel images containing the entire entorhinal cortex were collected using Visiopharm image capturing software approximately between Bregma -2.30mm and -4.50mm (Paxinos and Watson, 2009). For each image, the number of Iba-1+ cells was determined by Image Pro Plus software based upon both the size and immunoreactivity. This program has been shown to be comparable to the alternative method of visual

counts when immunoreactive cells are distinct from background (Francisco et al. 2004). The number of cells per section was averaged and expressed as Iba-1+ cells/section.

Hippocampal Iba-1+ cells were estimated by unbiased stereological methods, the optical fractionator, using the newCAST Stereology System (Visiopharm, Hoersholm, Denmark) installed on a Dell Precision 380 workstation coupled to the microscope. Following parameters similar to previous reports (Long et al. 1998), the DG, cornu amonis(CA)2/3, and CA1 regions of the dorsal hippocampus approximately between Bregma -2.30mm and -4.50mm as determined by Paxinos (Paxinos and Watson, 2009) were separately traced at 100x magnification. Section thickness was assessed at 600x using a 60x oil immersion lens and was averaged from three measurements taken at different locations within each region. The DG and CA2/3 were randomly sampled using a 70µm x 70µm counting frame with a 250µm x,y step length. The CA1 was randomly sampled using the same size counting frame and a 400µm x,y step length. After tissue processing, section thickness was approximately 24 µm, therefore, a dissector height of 20um with 2µm guard zones. Total Iba-1+ microglia in each region of interest was calculated using the following equation (West et al., 1991):

$$N = \sum Q \times \frac{1}{asf} \times \frac{1}{tsf} \times \frac{1}{ssf}$$

where Q is the number of cells counted, asf is the area sampling fraction (the counting frame: x,y step length ratio), tsf is the thickness sampling fraction (dissector height: section thickness ratio), and ssf is the section sampling fraction (the fraction of sections examined). For all stereological quantifications, coefficient of error ranged from 0.008 to 0.039 and averaged 0.021 ± 0.001. A coefficient of error less than 0.05 is considered adequate (Gundersen et al. 1999).

Enzyme Linked Immunosorbent Assay

Rats were rapidly decapitated and the brain immediately extracted. The hippocampus and entorhinal cortex were dissected on ice, snap frozen on dry ice, and stored at -80˚C until assayed. Thawed tissue was manually homogenized in an ice-cold lysis buffer (1mL of buffer/50mg of tissue; pH=7.4). All reagents used in the lysis buffer were purchased from Sigma (St. Louis, MO) unless otherwise noted. It consisted of 25mM HEPES, 0.1% 3-[(3-cholamidopropyl) dimethyl-ammonio]1-propanesulfonate, 1.3mM EDTA, 1mM EGTA, 10 µg/ml aprotinin, 10µg/ml leupeptin, 5mM $MgCl_2$ (Fisher, Fairlawn, New Jersey), 10 µg/ml pepstatin (Fluka, Milwaukee, WI), and 1mM PMSF (Fluka; Rabuffetti et al., 2000). Homogenates were centrifuged at 20,000 x *g* for 15 minutes at 4^{o}C and the supernatant stored at -80˚C. Total protein content was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Cytokine protein content was determined with an ELISA kit according to the manufacturer's instructions for rat TNF-α (Invitrogen product #KRC3011C, Camarillo, CA), IL-10 (Invitrogen product #KRC0101), IL-6 (R&D Systems product #R6000B, Minneapolis, MN), or TGF-β (Invitrogen product #KAC1688). All samples, standards, and positive controls were run in duplicate so that all tissue for one time point fit on one plate to reduce potential variability. Absorbance was measured at 450nm on a DXT880 Multimode Detector plate reader (Beckman Coulter, Brea, CA).The cytokine protein concentration was divided by the total protein concentration obtained in the BCA assay to correct for differences in tissue volume. Protein concentration is reported as pg of cytokine/ mg of protein.

Statistical Analyses

Data were analyzed and graphed using Prism Version 5.04 (GraphPad Software, Inc. La Jolla, Ca). All data are reported as the mean ± standard error of the mean and analyses considered significantly different if $p<0.05$. Behavioral scores were analyzed with a Kruskal Wallis test and BECs, autoradiography, OX-42, cytokine expression, and cell counts were analyzed by ANOVA with post-hoc tests as appropriate. Each region of the hippocampus or entorhinal cortex is considered independent and therefore was analyzed separately.

RESULTS

Animal Model Data

Intoxication parameters across all experiments were similar as shown in Table 2.4. The overall mean intoxication score for all ethanol animals was 1.9 ± 0.1 on the 6-point Majchrowicz scale, which indicates that all animals were, on average, "ataxic" immediately before dosing. This level of intoxication resulted in an overall mean dose of 9.2 ± 0.3 g/kg/day of ethanol and a BEC of 354.0 ± 7.5 mg/dL for all animals used. These parameters are similar to those reported in past studies with this model (Morris et al., 2010a; Nixon and Crews, 2004) and similar to that observed in voluntary consumption (Bell et al. 2009). Neither the Kruskal – Wallis (intoxication behavior) nor one-way ANOVAs (dose, BEC) showed differences in any intoxication parameter between ethanol groups at different time points.

Table 2.4 Experiment One Animal Model Data

Experiment	Group	Intoxication behavior (0–5 scale)	Dose (g/kg/day)	BEC (mg/dl)
Autoradiography	T0 (n=6)	1.8 ± 0.3	9.7 ± 1.3	318.0 ± 14.5
	T2 (n=6)	1.8 ± 0.3	9.8 ± 1.4	304.8 ± 18.4
	T4 (n=6)	1.8 ± 0.3	9.4 ± 1.6	336.2 ±19.7
	T7 (n=6)	1.8 ± 0.3	9.7 ± 1.4	345.1 ± 25.5
Immunohistochemistry	T0 (n=7-8)	2.0 ± 0.3	9.1 ±1.2	361.5 ± 17.2
	T2 (n=6)	1.9 ± 0.3	8.8 ± 1.5	286.7 ± 25.1
	T4 (n=6)	1.7 ± 0.3	9.3 ± 1.5	**
	T7 (n=7)	1.7 ± 0.2	9.8 ± 1.3	365.8 ± 36.4
	T28 (n=7-8)	2.0 ± 0.3	9.1 ± 1.6	332.9 ±26.0
ELISA	T0 (n=8)	1.9 ± 0.3	9.3 ± 0.9	331.3 ±23.3
	T1 (n=8)	2.1 ± 0.3	8.6 ± 1.3	401.5 ± 20.3
	T2 (n=7)	2.1 ± 0.3	8.8 ± 1.3	411.3 ± 14.5
	T4 (n=7)	2.2 ± 0.3	8.3 ± 1.6	400.5 ± 33.8
	T7 (n=7)	2.3 ± 0.3	8.3 ± 1.7	365.8 ± 36.4

*Table 2.4 Measures of various Intoxication parameters of the Majchrowicz model are statistically similar between time points among all experiments. **BECs from this group are omitted due to Analox malfunction but commonalities between behavioral intoxication measurement and dose suggest that the BECs should be comparable.*

[^{3}H]-PK-11195 autoradiography reveals early activation of microglia

Binding of the TSPO ligand, [^{3}H]-PK-11195, was measured by optical density at T0, T2, T4, and T7. Control levels of binding at each time point were not statistically different and therefore were pooled into a single control group (Readnower et al., 2010). As shown in representative images, ethanol treated animals have increased binding throughout the brain compared with controls (Figure 2.1). Specifically, one way ANOVAs showed a significant main effect of diet in each region of the hippocampus: CA1 [$F_{(4,27)}$ =14.93, p<0.0001], CA2/3 [$F_{(4,27)}$ =14.93, p<0.0001], and DG [$F_{(4,27)}$ =12.88, p<0.0001], as well as in entorhinal cortex [$F_{(4,27)}$ =9.08, p<0.0001]. Post-hoc Tukey's tests confirmed a significant increase (p<0.05) in the density of [^{3}H]-PK-11195 binding in each ethanol treated time point compared to controls in all regions examined (Figure 2.1).

Figure 2.1 Increased [^{3}H]-PK-11195 following EtOH Exposure

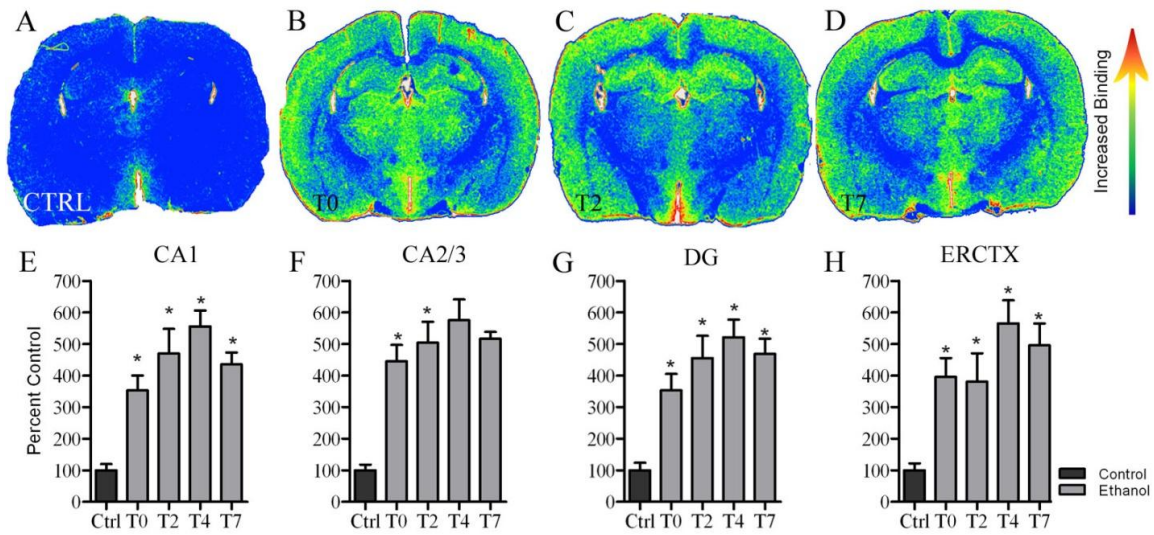


*Figure 2.1. [^{3}H]-PK-11195 upregulation following 4-day binge exposure. Representative false color autoradiographs depicting [^{3}H]-PK-11195 binding are shown for (A) controls (n = 8; black bars) as well as (B) ethanol (grey bars) at T0 (n = 6), (C) T2 (n = 6), and (D) T7 (n = 6). The legend in the top right corner shows how the false color reflects the intensity of binding. Quantitative analysis of the extent of binding are graphed for the (E) CA1, (F) CA2/3, (G) DG, and (H) entorhinal cortex. *p < 0.05.*

Immunohistochemical markers of microglia indicate partial activation phenotype

In order to see the earliest signs of activation, we examined OX-42 expression immediately after the last dose of alcohol (T0; rats are still intoxicated) and in a separate group after four weeks of abstinence (T28). OX-42 positive cells were apparent in both ethanol and control tissue which is consistent with its constitutive expression (Akiyama and McGeer, 1990). However, there was a visibly distinct increase in immunoreactivity at T0, reflecting a reduction in the ramification but a thickening of the processes in the ethanol animals compared with the controls (Figure 2.2). Two-way ANOVAs indicated a significant interaction between treatment and time point in the CA1 [$F_{(1,25)}$ =5.81, p=0.0236], CA2/3 [$F_{(1,26)}$ =5.71, p=0.0244] DG [$F_{(1,25)}$ =5.90, p=0.0227] fields, as well as in entorhinal cortex [$F_{(1,25)}$ =4.65, p=0.0409]. Planned post-hoc t-tests indicated a significant increase after ethanol exposure in all regions at T0: CA1 [$t_{(12)}$ =2.39, p=0.0345], CA2/3 [$t_{(12)}$ =2.23, p=0.0453], DG [$t_{(12)}$ =2.35, p=0.0367] and entorhinal cortex [$t_{(12)}$ =2.21, p=0.0472]. Although the contrast between ethanol and controls was not as distinct at T28, ethanol animals maintained a significant increase compared with controls in all regions except the DG: CA1 [$t_{(13)}$ =2.45, p=0.0288], CA2/3 [$t_{(13)}$ =2.25, p=0.0427], and entorhinal cortex [$t_{(13)}$ =4.80, p=0.0003].

The ED-1 antibody was used to recognize phagocytic microglia (Graeber and Streit, 2009), whereas the OX-6 antibody was used to visualize the upregulation of MHC-II. Neither ethanol nor control animals had ED-1 nor OX-6 positive cells within the parenchyma of the hippocampus or entorhinal cortex at T0, T2, T4, T7, or T28 (Figure 2.3). However, ED-1 and OX-6 positive cells were visible in blood vessels and along the meninges in both control and ethanol treated animals (Figure 2.3), similar to that previously reported in this model (McClain et al., 2011; Nixon et al., 2008). Thus, four-day ethanol treatment failed to induce phagocytic-stage microglia or increased MHC-II in the brain parenchyma at any time point.

Figure 2.2 CD11b (OX-42) upregulation following 4-day binge exposure.

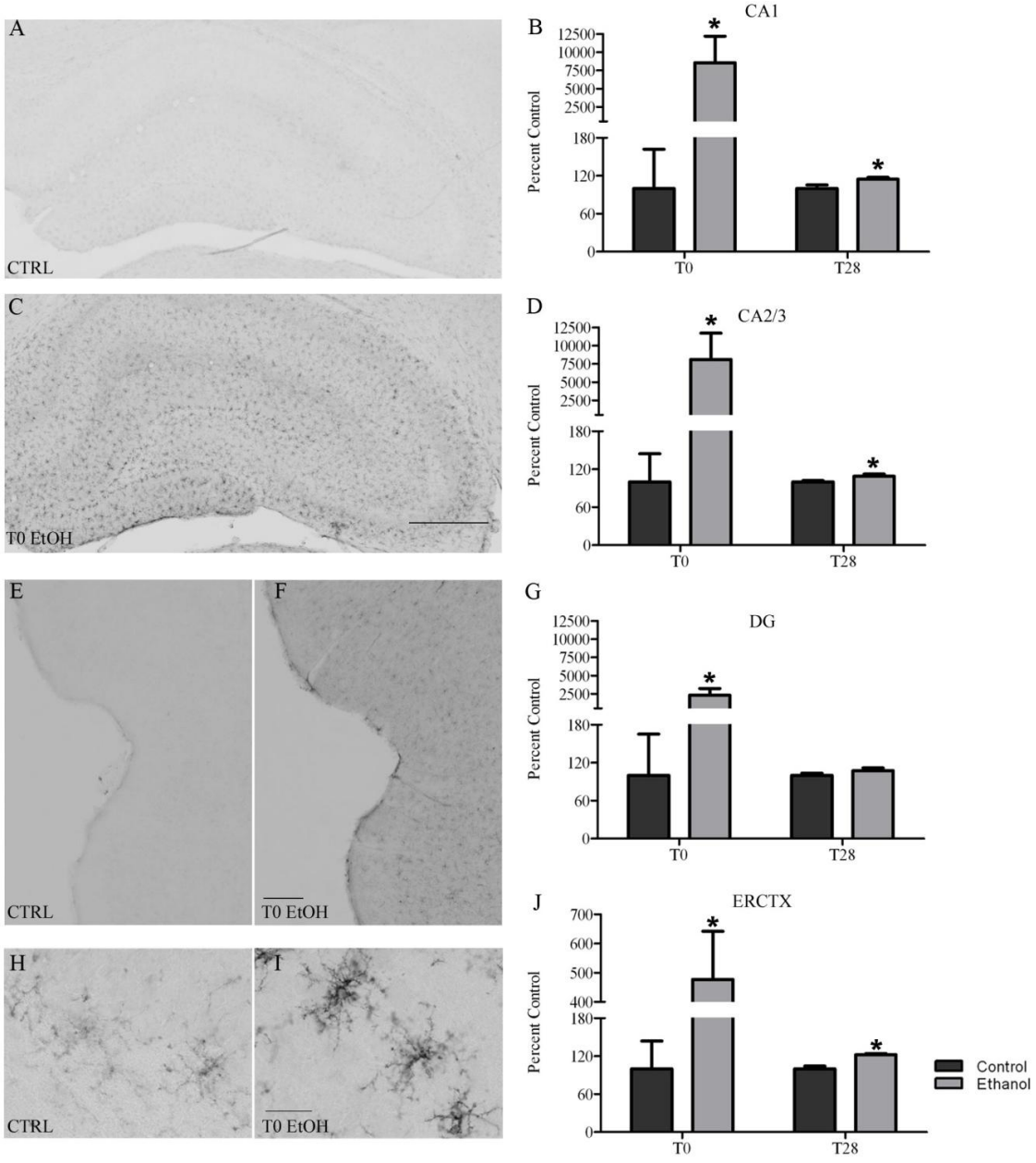


Figure 2.2. CD11b is upregulated in the hippocampus and entorhinal cortex at T0 as shown in representative photomicrographs in rats exposed to binge (C, F) ethanol (T0: n = 8; T28: n = 8; grey bars) compared to (A, E) controls (T0: n = 7; T28: n = 7; black bars). Higher magnification of microglia seen in the hippocampus is shown for both (H) control and (I) ethanol. Quantifications of OX-42 immunoreactivity for the subregions of the hippocampus were significantly different: (B) CA1, (D), CA2/3, and (G) DG as well as the (J) entorhinal cortex. Scale bar in C = 500 µm; F = 300 µm; J = 10 µm. *p < 0.05.

Figure 2.3 No OX-6 or ED-1 Positive Microglia

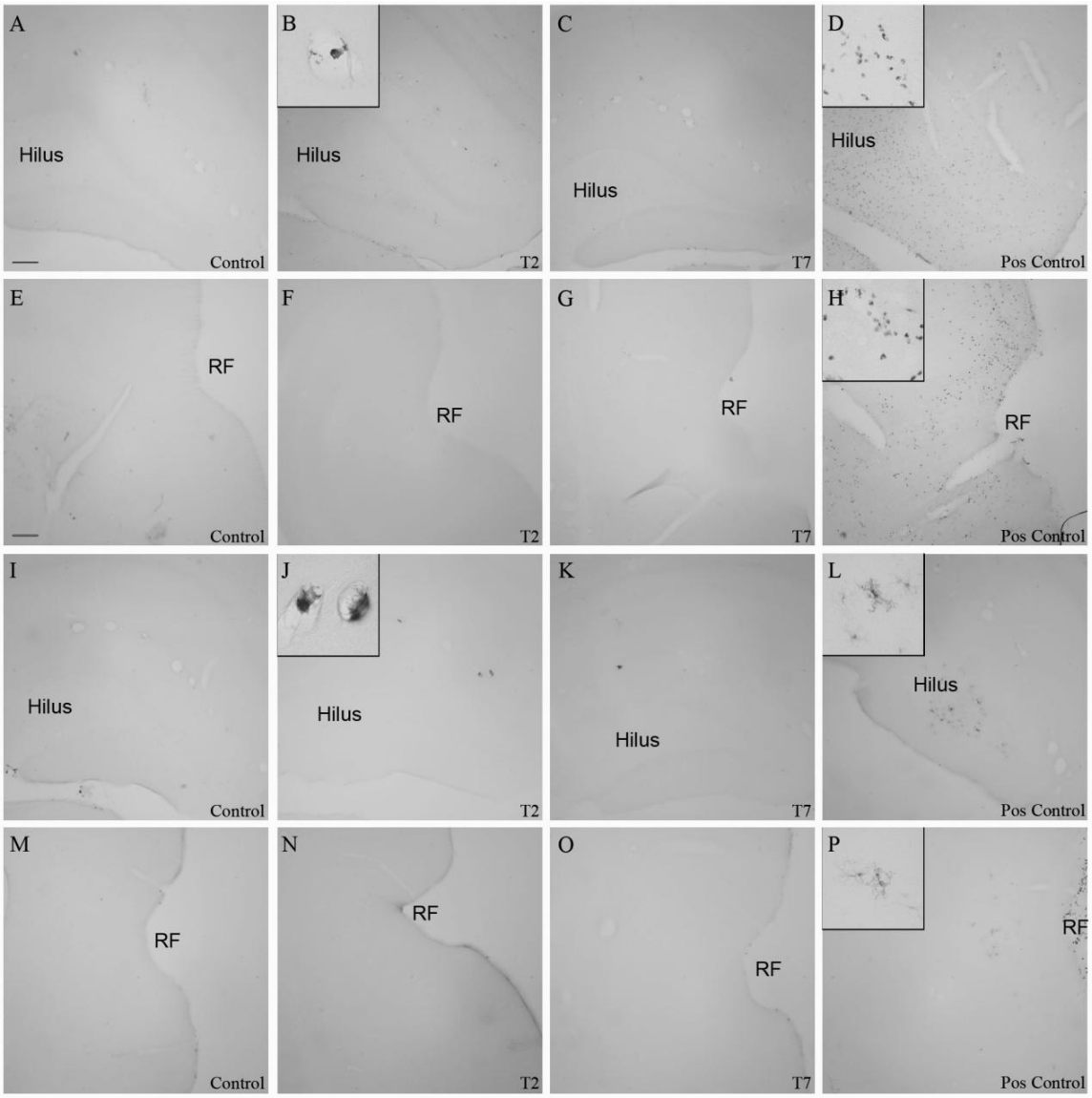


Figure 2.3. ED-1 was not visible in the (A–D) hippocampus or (E–H) entorhinal cortex as seen in representative photomicrographs for (A, E) controls (T2: n = 7; T7: n = 8) or (B, C, F, G) ethanol (T2: n = 6;T7: n = 7) rats. No OX-6 positive cells were visualized in the (I-K) hippocampus or (M-O) entorhinal cortex as seen in representative images for (I, M) controls or ethanol rats at (J,K, N, O. Phagocytic and immune responsive macrophages were visible in the blood vessels as seen in insets of (C) ED-1 and (J) OX-6, respectively. ED-1 and immunopositive cells were visible in the (D, H, L, P) positive control tissue from a rat treated with kainic acid. RF = rhinal fissure. Scale bar = 150 μm.

Microglia proliferation results in increased number

We have previously shown that microglia proliferate two days after a four-day alcohol binge (McClain et al., 2011; Nixon et al., 2008); therefore stereological estimates of Iba-1-positive microglia were conducted at seven (T7) and twenty eight (T28) days following the last ethanol dose in the hippocampus. The total number of microglia was increased in the hippocampus of ethanol treated animals compared with controls seven days after ethanol exposure (T7; Figure 2.4). Two-way ANOVAs indicated a significant main effect of diet (CA1 [$F_{(1,23)}$ =14.39 p=0.0009], CA2/3 [$F(_{1,23)}$ =12.14 p=0.0020], DG [$F_{(1,23)}$ =12.16 p=0.0020]), time (DG [$F_{(1,23)}$ =10.88 p=0.0031]), and a significant interaction between diet and time in the CA1 [$F_{(1,23)}$ =4.37 p=0.0477], and DG [$F_{(1,23)}$ =13.32 p=0.0013]. Planned post-hoc t-tests indicated a significant increase after ethanol exposure in all regions of the hippocampus at T7: CA1 [$t_{(10)}$ =3.22, p=0.0092], CA2/3 [$t_{(10)}$ =2.28, p=0.0457], and DG [$t_{(10)}$ =5.038, p=0.0005] However, by T28, the number of hippocampal microglia returns to control levels in all regions except the CA2/3 [$t_{(13)}$ =2.66, p=0.0195]. In the entorhinal cortex, microglial cell number was estimated by an automated cell count, where no change was seen in the number of microglia between ethanol (586.5±55.4 microglia/section, n=7) and control animals (623.3±26.7 microglia/section, n=7) at T7, therefore no further time point was examined.

Figure 2.4 Increase in microglia number following 4-day binge exposure

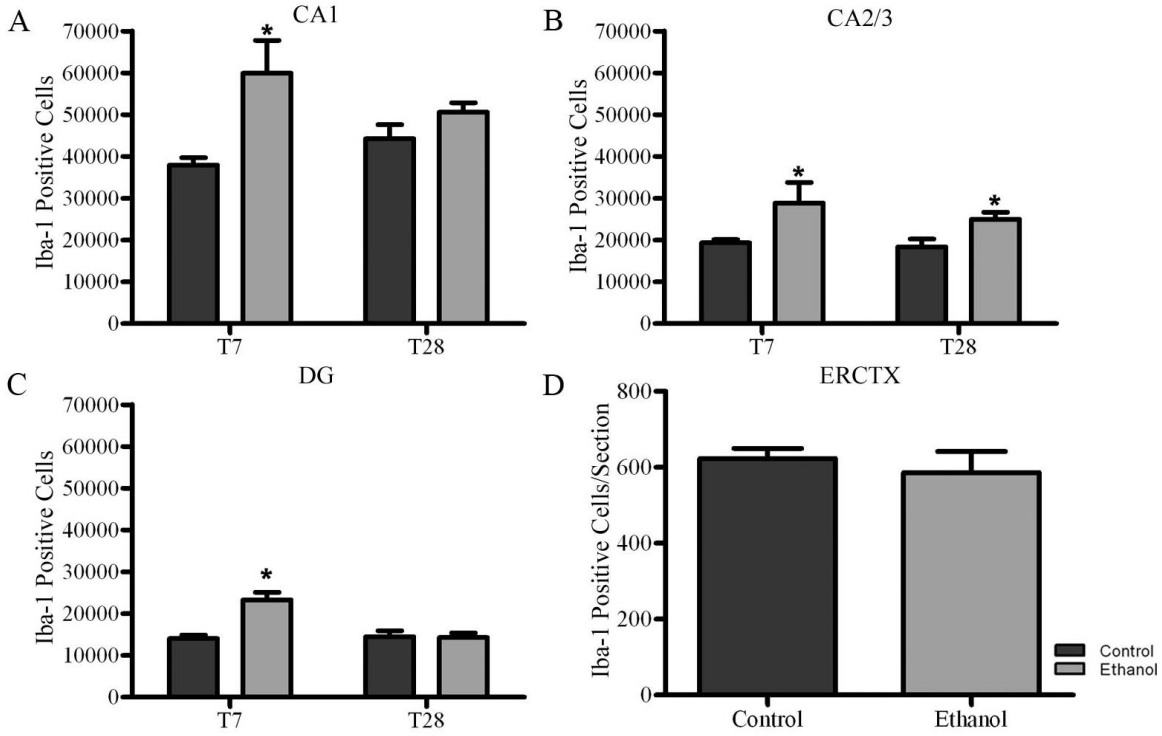


Figure 2.4. Stereological estimates indicate an increase in the number of microglia in ethanol treated animals (n = 7; grey bars) compared with control (n = 8; black bars) at T7 in the (A) CA1 (B) CA2/CA3, and (C) DG. This increase persists twenty-eight days later in the (B) CA2/3 in ethanols (n = 7) compared with controls (n = 7). There was no difference in cell counts determined by image analysis between ethanols and controls at T7 in the (D) entorhinal cortex. *p < *0.05.*

Cytokine expression also suggests low grade activation phenotype

In order to assess the functional state of microglia, cytokine levels were assayed via ELISA. Increases in the proinflammatory cytokines IL-6 and TNF-α , are associated with classically activated microglia, but not partially activated microglia, and can be used to differentiate the two phenotypes of microglia (Table 2.1). IL-6 is a proinflammatory cytokine secreted by activated microglia in response to brain injury but can also act in an autocrine function to stimulate surrounding microglia into a phagocytic state (Chiang et al. 1994; Woodroofe et al. 1991). Two-way ANOVA's showed a main effect of time in the hippocampus [$F_{(4,59)}$ =8.18, p<0.0001], but Bonferroni corrected post-hoc t-tests showed no statistical difference between ethanol and control animals in the region. However in the entorhinal cortex, two-way ANOVA indicated a significant main effect of diet [$F_{(1,54)}$ =7.13 p=0.01], time [$F_{(4,54)}$ =2.88 p=0.03], and a significant interaction between diet and time point [$F_{(4,54)}$ =4.72 p=.002] (Figure 2.5). Bonferroni corrected post-hoc t-tests show a significant 36% decrease [$t_{(11)}$ =3.97, p=0.011] in IL-6 in ethanol animals compared to controls in the entorhinal cortex at T2. Taken together, these results indicate that inhibition of basal IL-6 expression occurs after ethanol withdrawal in a temporally and regionally specific manner. In addition to IL-6, TNF-α is a proinflammatory cytokine expressed by fully activated microglia and increased after many forms of injury (Vitarbo et al., 2004). Two-way ANOVA of the hippocampus showed a main effect of time [$F_{(4,63)}$ =20.77, p<0.0001], but there was no statistical differences between ethanol and control animals after Bonferroni corrected post-hoc t-tests. Despite significant main effects of both diet [$F_{(1,54)}$ =4.77 p=0.03], time [$F_{(4,59)}$ =8.86 p<.0001] in the entorhinal cortex, Bonferroni corrected post-hoc t-tests indicated no difference between ethanol and control animal at any time point. This lack of TNF-α

upregulation in brain is consistent with previous reports in rats (Ehrlich et al., 2012; McClain et al., 2011; Zahr et al., 2010), but not mice (Qin et al., 2008).

Figure 2.5 No Increased proinflammatory cytokine expression in the 4-day binge.

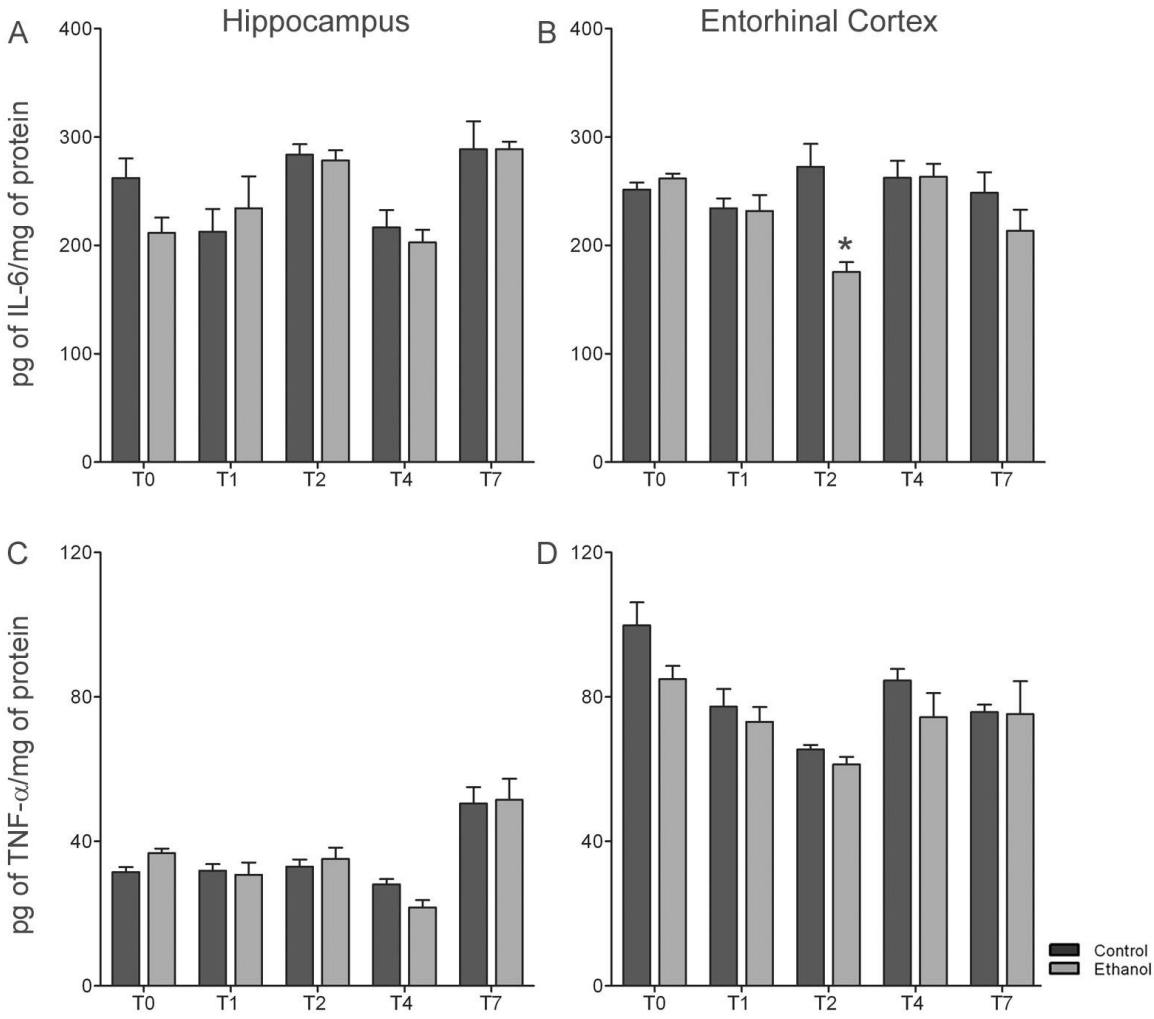


*Figure 2.5. Concentrations of (A, B) IL-6, (C, D) TNF-α were determined by ELISA in both the hippocampus (A, C) and entorhinal cortex (B, D). A 36% decrease of IL-6 was measured in the (B) entorhinal cortex at T2 in ethanol animals (n = 7; black bars) [175 pg/mg ± 8.9] compared to controls (n = 7; grey bars) [272 pg/mg ± 21.2]; however, no change in TNF-α was seen in either the (E) hippocampus or the (F) entorhinal cortex. *p < 0.05.*

Basal expressions of TNF-α and IL-6 were not increased following four-day ethanol exposure, suggesting the lack of a proinflammatory response. Therefore, we examined the effects of ethanol on the growth factor, TGF-β, as well as IL-10, an anti-inflammatory cytokine (Fiorentino et al. 1991; Polazzi et al. 2009). A significant interaction between diet and time point was shown in the hippocampus using a two-way ANOVA of TGF-β [$F_{(4,53)}$ =4.20 p=0.005]. Bonferroni corrected post-hoc t-tests revealed a significant 26% increase [$t_{(11)}$ =2.673, p=0.0434] in TGF-β in ethanol animals compared to controls at T7 (Figure 2.6). Despite a significant main effect of time point in the entorhinal cortex [$F_{(4,47)}$ =18.65 p<0.0001], no difference in TGF-β was observed between ethanol and control treated animals. In the hippocampus, a two-way ANOVA of IL-10 concentrations indicated a main effect of time point [$F_{(4,59)}$ =6.71 p=0.0002], plus a significant interaction between treatment and time point [$F_{(4,64)}$ =3.24, p=0.01]. Bonferroni corrected post-hoc t-tests revealed a significant 26% increase [$t_{(11)}$ =3.97, p=0.011] in IL-10 in ethanol animals compared to controls in the hippocampus at T7 (Figure 2.6). A two-way ANOVA showed no statistically significant main effects or interaction between diet and time point in the entorhinal cortex indicating no significant difference in the mean protein concentration between ethanol treated animals and controls (Figure 2.6).

Figure 2.6 Increased TGF-β and IL-10 expression after 7 days of abstinence.

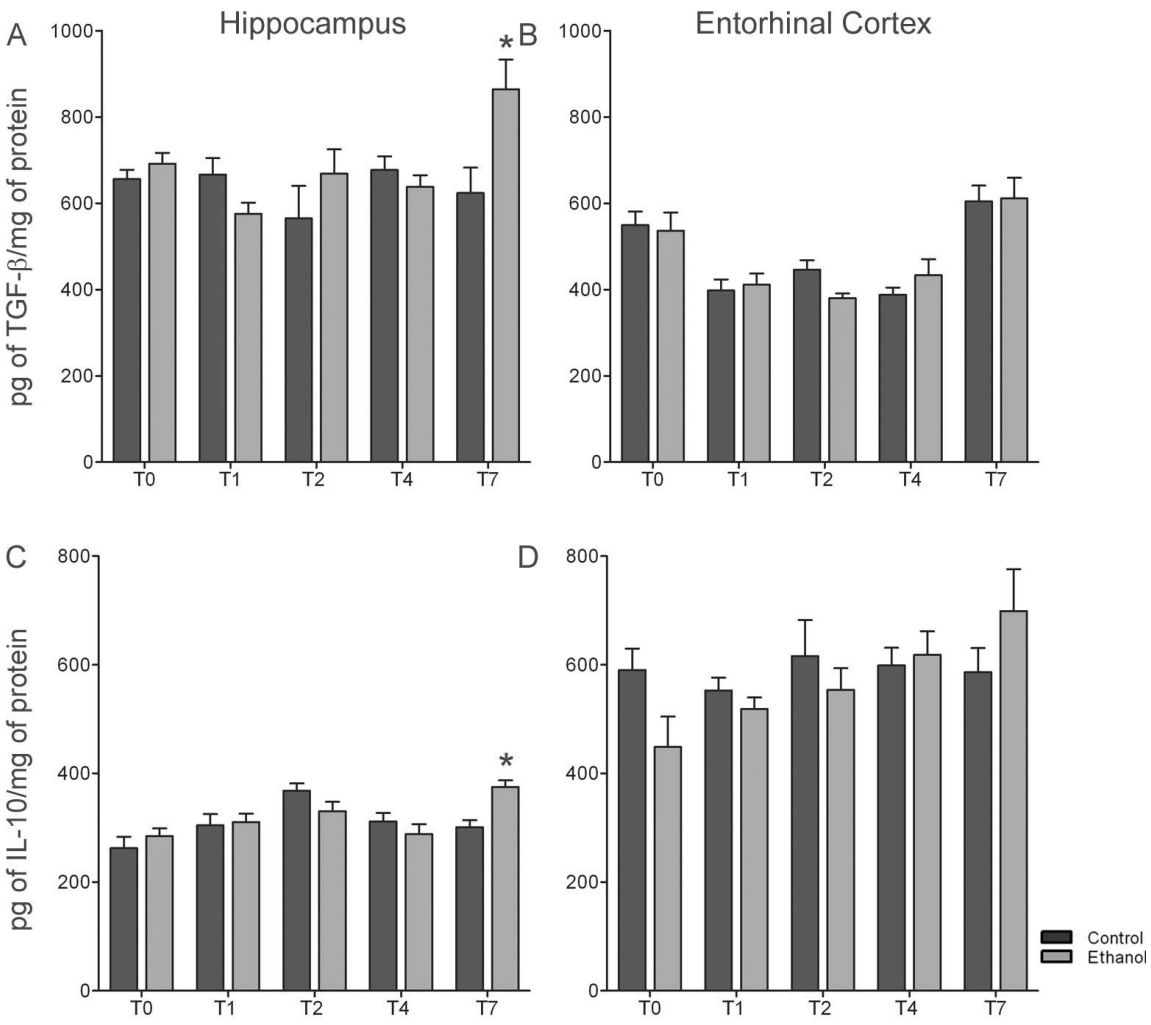


Figure 2.6 Concentrations of (A, B) TGF-β (C, D) IL-10 were determined by ELISA in both the hippocampus (A, C) and entorhinal cortex (B, D). An increase in both (A) TGF-β (38%) (C) IL-10 (26%) was seen in ethanol animals (n = 6,7 respectively; grey bars) compared with controls (n = 7; black bars) in the hippocampus at T7. *p < *0.05.*

BBB remains intact following four-day binge ethanol exposure

In order to assess, whether the BBB is possibly breached by four-day binge ethanol exposure, we examined the penetration of IgG molecules during intoxication and at T2. Penetration of IgG into the parenchyma was observed in the ventral hypothalamus around the 3rd ventricle, a region known to lack an intact BBB under physiological conditions (Schmidt and Grady 1993). However, qualitative analysis of IgG immunoreactivity between Bregma -2.30mm and -4.50mm (Paxinos and Watson, 2009) showed that both ethanol and control animals had few, if any IgG positive cells or diffusion in the parenchyma of either the hippocampus or entorhinal cortex at T0 or T2 (Figure 2.7). Therefore, the BBB does not appear to be breached in this model.

Figure 2.7 No disruption in the BBB.

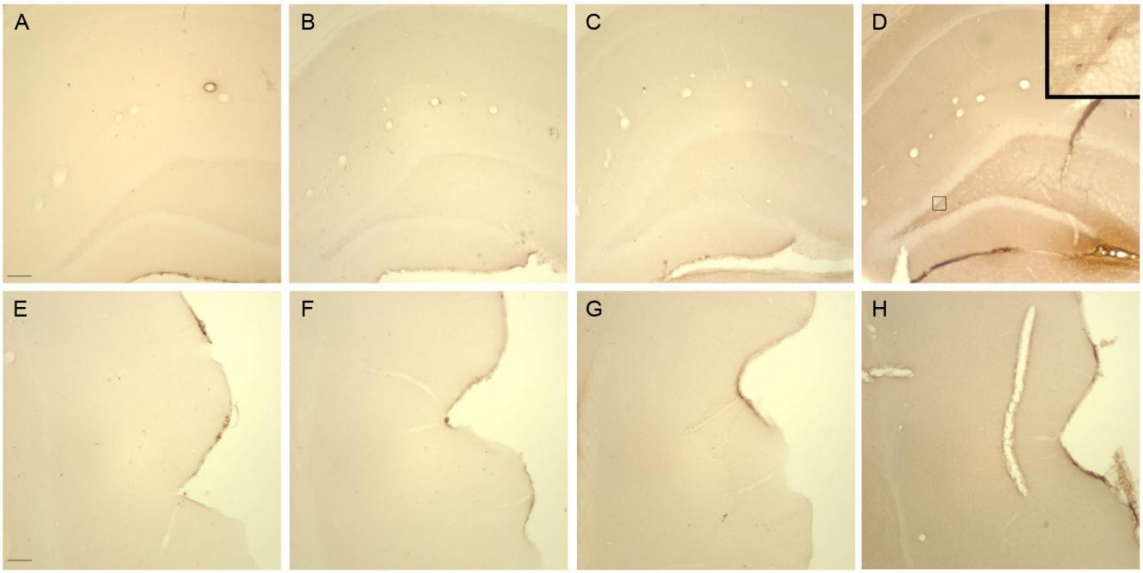


Figure 2.7 There is no disruption in the BBB following ethanol as there is little to no IgG staining in either the (B, C, F, G) ethanol (T0: n = 8; T2: n = 6) or (A,E) control (T0 n = 6; T2 n = 7) compared with a (D,H) kainate positive control. Scale bar = 400 µm.

DISCUSSION

Microglia take on a variety of phenotypes, which can be used to predict the cell's role in brain insult or neurodegenerative disease. The major finding of this work is that both morphological and functional evidence from these experiments support the conclusion that binge ethanol exposure does not classically activate microglia and is consistent with definitions of partial activation. The lack of classically activated microglia therefore does not meet the criteria for classical definitions of inflammation. Of Raivich's five levels of microglial activation (Raivich et al., 1999a), these data support that four-day binge ethanol exposure only appears to activate cells up to stage 2. A step-wise progression is noted beginning while the animals are intoxicated (T0) where stage 1 (Table 2.1) or low level "alert" activation begins to occur and persists for at least twenty eight days according to [^{3}H]-PK-11195 autoradiography for the TSPO receptor and OX-42 (CR3) immunoreactivity. Both markers are upregulated during and after four-day binge alcohol exposure. In addition, the morphology of OX-42 positive cells in ethanol-exposed brains supports that microglia are "alert" and "homing" as they appear less ramified with thicker, bushier processes (Figure 2.2). A stage 2 level of activation, or "proliferation and homing," was suggested previously with the observation of proliferating microglia (Nixon et al., 2008). That microglia proliferate and home to sites of damage is further supported by the increased numbers of Iba-1+ microglia observed at T7 in all regions of the hippocampus, which persists in the CA2/3 at T28 (Figure 2.4). Importantly, the highest indices of activation, proliferation and increased number, are observed well after the peak of alcohol-induced cell death during intoxication (Crews 2000; Kelso et al. 2011), which suggests that alcohol-induced microglial activation is a consequence of alcohol-induced cell death.

However, neither TSPO nor CR3 upregulation indicates the level of activation. Therefore, in order to determine microglia phenotype, more classical markers of full

activation were evaluated. Neither OX-6 nor ED-1 were detected in the brain parenchyma, which indicates that few, if any, microglia have been activated to either a phagocytic or bystander activation state (Kato et al. 1995). Indeed, with the addition of these data, ED-1 has been exhaustively examined following four-day binge ethanol exposure, the most acutely damaging model of an AUD, and at no time point examined have ED-1-positive cells ever been found inside the brain parenchyma (McClain et al., 2011; Nixon et al., 2008). Therefore, morphology, number and marker data converge to support that microglia are only partially activated, specically to at least stage 2 in the hippocampus and to stage 1 in the entorhinal cortex.

Activated microglia not only change morphologically but also functionally as they secrete cytokines and growth factors that may impact the surrounding environment. Similarly, these cytokines can have either damaging or protective/reparative effects depending on the phenotype or level of microglial activation (Raivich et al. 1999a; Suzumura et al. 2006). Therefore, we examined key cytokines at critical time points of previously reported cellular events following four-day binge exposure. Cytokine expression following binge ethanol exposure also indicated that microglia are only partially activated. Proinflammatory TNF-α was not changed at any time point, IL-6 was selectively decreased at T2 in entorhinal cortex, the time of microglial proliferation, whereas anti-inflammatory cytokines, IL-10 and TGF-β, which can be secreted by alert/homing microglia, were selectively increased at T7 in the hippocampus. Partially activated microglia secrete both TGF-β and IL-10, and are known to suppress microglia activation and subsequent neuronal damage (Ledeboer et al. 2000; Sharma et al. 2011; Spittau et al. 2012). The increase IL-10 and TGF-β seven days after ethanol exposure (T7) in the hippocampus comes after significant neuronal damage in this region and, intriguingly, coincides with reactive neurogenesis (Kelso et al., 2011 Nixon and Crews,

2004; Obernier et al., 2002a). However, TNF-α and IL-6, released in the highest levels of activation, were not increased at any time point in either the hippocampus or entorhinal cortex (Bethea et al. 1999; Stoll et al. 2000). The lack of effect on TNF-α is consistent with recent reports from multiple laboratories that TNF-α is not increased in rats following excessive alcohol exposure (Ehrlich et al., 2012; McClain et al., 2011; Zahr et al., 2010), though conflicts with reports in mice (Alfonso-Loeches et al. 2010; Qin and Crews 2012a; Qin et al. 2008). It is important to note that the source of these cytokines was not determined in the present study or the cited reports as reactive astrocytes also secrete many of the same cytokines (Lau and Yu 2001). Astrocytes are activated in the four-day binge model used and other alcohol models, though in a more delayed time course than that observed for microglia (Kelso et al., 2011). Because of the overlap in microglia and astroglia activation at T7 in this model, it is impossible to definitively link microglia activation with the secretion of particular cytokines. An important future discovery will be to show the cellular source of these cytokines *in vivo*. In summary, cytokine expression patterns following four-day binge alcohol exposure are consistent with that observed in immunohistochemical and morphological analyses – microglia phenotype is not one of classical activation, but merely partial activation.

The activation state of microglia is critical to understanding their role in alcoholic neuropathology. Microglia progress stepwise through these various phenotypes, each of which is predictive of the cell's role in homeostasis/neuroprotection versus neurodegeneration (Raivich et al. 1999a; Schwartz et al. 2006; Vilhardt 2005). Although the concept of a graded state of activation (phenotype) has resolved the debate as to whether microglia are "good" or "bad," each insult still results in a distinct response (Harting et al., 2008; Lai and Todd, 2008; Saijo and Glass, 2012). Even various patterns of alcohol intake produce a distinct response. As shown here, four-day binge ethanol

exposure, which is an acutely damaging event compared to more chronic models, only produces partially activated microglia. Partially activated or low level phenotypes are more closely associated with roles in homeostasis and neuroprotection and therefore alcohol-activated microglia may be playing a role in neuroprotection, repair, or in the hippocampal DG, regeneration (Battista et al. 2006; Engelsberg et al. 2004). Although it may seem surprising that a brain insult as severe as high blood alcohol concentrations and alcohol-induced neurodegeneration, does not result in an overt, phagocytic level of reactive microgliosis, not all types of brain injury result in a full phagocytic, i.e. classical, microglial response (Graeber et al. 1998). Indeed, a recent report details phagocytosis independent of fully activated microglia (Sierra et al. 2010) and multiple reports show that partially activated microglia are necessary in neuroprotection and axonal regeneration (Shokouhi et al. 2010; Wainwright et al. 2009).

Intriguingly, intermittent exposure to ethanol results in evidence of more classically activated microglia such as TLR4 upregulation (Alfonso-Loeches et al. 2010; Fernandez-Lizarbe et al. 2009). Greater levels of activation with intermittent exposure models leads us to speculate that the initial exposure may serve as a priming stimulus to microglia such that subsequent exposures result in over-response as seen in other neurodegenerative disease models (Bilbo and Schwarz 2009; Perry et al. 2003). The concept of microglia priming would explain why more classic-like activation is observed with multiple exposures or multiple intoxication/withdrawal cycles as that used by Qin (Qin et al., 2008), as opposed to our single cycle of prolonged intoxication then withdrawal and why the pattern of drinking is more associated with gliosis than the level of consumption (Riikonen et al. 2002). Unfortunately, these and other data support that microglia remain “primed” or partially activated for long periods of time after exposure. For example, [^{3}H]-PK-11195 remains upregulated months after alcohol exposure

(Obernier et al. 2002b; Syapin and Alkana 1988) and the number of microglia remains increased at least a month after the binge in some regions (Figure 2.4). The long-term persistence of some level of activation supports the theory that cells could be "primed" by the initial damaging binge exposure. Furthermore, repeated cycling could also change the microglia response to secondary neuroimmunomodulators such as systemic inflammation (Qin et al., 2008; Zahr et al., 2010) which could be crucial when considering the large number alcoholics have systemic inflammation associated with liver disease (Polednak 2012; Seth et al. 2011; Wang et al. 2012b). This observation is important clinically as human binges tend to occur in an episodic nature and binge-pattern drinkers have a greater likelihood of neurodegeneration (Hunt, 1993). Thus, our data is consistent with the idea that an initial "hit" of binge-induced damage appears to partially activate microglia as a consequence of damage, but if this partial activation primes microglia, secondary "hits" or binge exposure could "polarize" or result in a more classical activation phenotype and/or inflammation. Although this study did not address polarization of microglia, nor the specific definitions associated with alternative or M2 activation, this could be a logical next step of the current work. A defining hallmark of classical inflammation is a compromised BBB, which, based on an examination of IgG expression, is not evident in the four-day binge model, the most severe of AUD models. Indeed, these data agree with evidence from less acutely damaging but longer term, chronic models of exposure such as 12-month 20% ethanol in the drinking water (Ehrlich et al., 2012). Other alcohol models, that have enhanced proinflammatory cytokine expression, do show BBB disruption, further supporting the theory that BBB disruption is necessary for a true neuroinflammatory event (Abdul Muneer et al. 2012). Importantly, the lack of evidence for a BBB compromise in this model strongly supports that classical inflammation does not occur with four-day binge exposure. Although this is only one

model of an AUD, the well-defined cell death and degeneration profile coupled with data reported here does not indicate that classical inflammation drives alcohol-induced brain damage or that inflammation, according to classical definitions, occurs at all in this model.

The timecourse of expression of these various microglial markers and cytokine effects coupled with published timecourses of alcohol-induced cell death in this model (Crews et al., 2000; Kelso et al., 2011) support that alcohol-induced microglia activation is a *consequenc*e, *not a cause* of alcohol neurotoxicity. Alcohol-induced partial activation suggests a beneficial role of microglia in this model of an AUD, especially as no reports to date have observed fully activated, phagocytic microglia in brains from alcoholics. Indeed, if you remove microglia in many forms of neurodegeneration, worsened outcomes occur (Wainwright et al., 2009). Microglia have diverse roles in homeostasis, including newly defined roles in synaptic plasticity and neurotransmission (Tremblay and Majewska 2011) and it is not known, nor revealed by these data, how partial activation might affect their homeostatic actions in synaptic plasticity. Intriguingly, the lack of phagocytic microglia could have implications for synaptic pruning and remodeling, especially in ongoing neurogenesis in the DG (Tremblay and Majewska, 2011). Thus, the inflammation hypothesis of AUD and targeting microglia in the treatment of AUDs must be considered with caution. Neuroinflammatory responses alone do not lead to AUDs and many of the reported microglial activation markers are expressed in the beneficial partially activated or acquired deactivated microglia that help to resolve and repair damage (Colton and Wilcock, 2010). Thus, it is not just that these microglia are activated by excessive alcohol exposure; the critical information is their phenotype. Therefore, these data do not rule out a role for microglia in AUDs, but do not support a direct relationship between alcohol, microglial activation and inflammation

driven neurotoxicity. Careful consideration of these various current and previous studies, however, suggest that this partial activation phenotype could be consistent with a “primed” state such that repeated bouts of damaging, excessive alcohol intake, which is consistent with binge-benders in AUDs, may eventually result in highly or classically activated microglia and a proinflammatory state. The immediacy of microglial activation during alcohol intoxication, which was observed here, suggests that controlling the activation state of microglia during ethanol exposure may be a potential therapeutic target for AUDs. If microglia can be limited to only partial activation, perhaps they may be beneficial to endogenous repair systems after alcohol-induced neurodegeneration.

Chapter 3: Early evidence of microglial activation in an alcohol-induced neurodegeneration model

INTRODUCTION

Excessive consumption of ethanol, one of the key characteristics of an AUD, can result in neurodegeneration in the corticolimbic pathway of human alcoholics and has been associated with a variety of cognitive deficits (Beresford et al. 2006; Parada et al. 2011; Pfefferbaum et al. 1992; Sullivan et al. 1995). In fact, alcohol-induced cognitive impairments are the second leading cause of dementia, behind only Alzheimer's Disease (Eckardt and Martin 1986). One mechanism that has been proposed to cause alcohol-induced neurodegeneration is neuroinflammation (Crews 2012). This mechanism has been inferred from the brains of post-mortem alcoholics that have increased microglial activation (He and Crews 2008) as well as modulations of transcriptions factors associated with innate immune gene induction like NF-kB (Okvist et al. 2007). However, recent studies using the Majchrowicz model of an AUD, which consistently shows alcohol-induced neurodegeneration (Collins et al. 1996; Kelso et al. 2011; Obernier et al. 2002b), have proposed that microglial activation following this exposure is not inflammatory but alternatively has a beneficial phenotype that may be involved in homeostatic mechanisms (Marshall et al. 2013; McClain et al. 2011; Zahr et al. 2010a). Furthermore, studies using the Majchrowicz AUD model suggest that microglial activation is in response to neuronal damage and not the cause (Marshall et al. 2013; McClain et al. 2011). These studies focused on alcohol microglial effects in snapshots during recovery following the four-day binge model; however, it has been shown that alcohol-induced brain damage occurs earlier during the binge exposure (Hayes et al. 2013; Obernier et al. 2002a). Because microglia respond quickly to environmental perturbation (Nimmerjahn et al. 2005), it is perceivable that microglial activation would occur concurrently with neurodegeneration and well before the end of

the four-day binge paradigm. Furthermore if microglia activation is a driving force of neurodegeneration in this model, microglia activation would occur before evidence of cell death seen after just two days of exposure (Hayes et al. 2013; Obernier et al. 2002a). This study examines how early this activation occurs within the Majchrowicz AUD model.

Determining the immediacy of microglial activation is an important factor in understanding their role within AUDs and alcohol-induced neurodegeneration. Early, immediate activation and neuroinflammation are necessary defense mechanisms in response to damage. Acute microglial activation has been described as vital for "housekeeping" (Nimmerjahn et al. 2005) and "nursing" (Streit 2002b) in the CNS. If microglia are not responding after immediate signs of damage, it could indicate that microglial function is compromised. Furthermore, modulation of neuroinflammatory pathways has been proposed as a potential therapeutic for alcohol-induced brain damage (Crews 2008), and other neurodegenerative disorders have shown determining the timing of immune modulation is crucial for therapeutic outcomes (Ceulemans et al. 2010; Kriz 2006).

The current studies examine the immediacy of the microglial response using [^{3}H]-PK11195 binding, a sensitive marker of microglial activation. [^{3}H]-PK11195 binding was measured following various durations of ethanol exposure. Stereological estimates of microglia cell numbers were used to help interpret [^{3}H]-PK11195 binding results as densitometric analysis can be convoluted by changes in cell number.

MATERIALS AND METHODS

Experimental Model of an AUD

All included procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee as well as Guidelines for the Care and Use of Laboratory Animals (NRC, 1996). Male Sprague-Dawley rats (n=51; Charles River

Laboratories, Raleigh, NC) arrived at 275-300g and were allowed five days for acclimation to single housing conditions in a University of Kentucky AALAC accredited vivarium with a 12h light:dark cycle. During the acclimation period, animals were handled for three days and had *ad libitum* access to food and water.

Rats were divided into three groups and subjected to a modified version of the Majchrowicz model of an AUD for either 1, 2, or 4 days (Hayes et al. 2013; Majchrowicz 1975; Morris et al. 2010b). This model has previously been described in chapter two, and the binge methods used in these experiments were identical outside of the number of days of exposure. Rats were euthanized within hours of the last dose of ethanol or control diet. BECs were determined from blood taken following the last dose of ethanol for animals exposed to one or two days of ethanol but following the seventh dose of alcohol for animals with four days of exposure as described in chapter two. Samples were centrifuged to obtain serum and stored at -20˚C. BECs were determined from triplicate runs of serum using an AM1 Alcohol Analyser with a 300mg/dL external standard for calibration (Analox, London, UK). The average BECs were reported as mg/dL.

Autoradiography

Changes in the expression of the mitochondrial translocator protein 18kDa (TSPO) were measured using densitometric analysis of [^{3}H]-PK11195 binding. This radioligand was used to assess microglial activation following one or two days of ethanol because of its high sensitivity in determining activation (Benavides et al., 2001; Readnower et al., 2010). Autoradiography was conducted as described in previously (Kelso et al. 2006; Sparks and Pauly 1999) and are identical to chapter two that showed upregulation of TSPO after four days of exposure in this model (Marshall et al. 2013). However, the control animals in these studies were not collapsed for analysis. The

relative binding was determined using ImageJ software and expressed as percent control.

Immunohistochemistry

Immunohistochemical techniques were similar to that previously reported (Marshall et al. 2013; McClain et al. 2011). Rat euthanization, brain extraction, and tissue treatments were identical to that stated in chapter two. Every twelfth section was used in an immunohistochemical staining process using an antibody against Wako, Richmond, VA). This Iba-1 antibody recognizes a calcium binding protein that is specifically found in microglia (Heizmann and Hunziker, 1991; Imai et al., 1996; Ito et al., 1998). Iba-1 is present in microglia regardless of phenotype but is upregulated upon activation (Donato, 1999; Donato, 2003; Hwang et al., 2006). Immunohistochemical procedures were identical to that previously described in chapter two. Tissue was mounted onto slides and coverslipped using Cytoseal® (Stephens Scientific, Wayne, NJ).

Quantification of Iba-1 Cells

Slides were coded so that experimenters were blinded to the treatment group during quantification. Unbiased stereological methods were used to estimate the number of Iba-1+ cells in the subregions of the hippocampus using the newCAST Stereology System (Visiopharm, Hoersholm, Denmark) installed on a Dell Precision 380 workstation coupled to an Olympus BX-51 microscope (Olympus, Center Valley, PA). The stereological methods used were identical to our previous report (Marshall et al. 2013) as described in chapter two. For all stereological quantifications, coefficient of error ranged from 0.010 to 0.037 and averaged 0.023 ± 0.001 (Gundersen et al., 1999).

Image Pro Plus, an image analysis system that has been shown to be a valid alternative method for determining cell number (Francisco et al. 2004), was used to

quantify Iba-1+ cells in the entorhinal cortex (Marshall et al. 2013). The methods used were identical to that reported in chapter two. The number of cells in each section was averaged and expressed as Iba-1+ cells/section .

During stereological estimates, the appearance of dystrophic microglia was noted in the molecular layer of the DG. Dystrophic microglia have a distinct morphology compared with other microglial phenotypes. Dystrophic microglia have cytorrhexis or cytoplasmic fragmentation as well as beaded processes (Streit et al. 2009). Therefore, the number of dystrophic microglia was determined within the molecular layer of the DG where this phenomenon was initially observed. Microglia were characterized as being dystrophic if they possessed fragmented cell bodies and had the appearance of beaded processes (Streit et al. 2004b). Dorsal hippocampal sections stained with Iba-1 between Bregma -2.30mm and -4.50mm were examined for the dystrophic characteristics. Profile counting methodology was performed using a 60x oil immersion lens due to the infrequency and inhomogeneous distribution of these cells (Morris et al. 2010a; Popken and Farel 1997). Counts are expressed as cells/section.

Enzyme Linked Immunosorbent Assay

Because our previous studies indicate that microglia are not classically activated in this model, only brain derived neurotrophic factor (BDNF) was assessed after four days of ethanol exposure. Although not exclusively secreted by microglia, enhanced BDNF expression by microglia can afford neuroprotection while decreases are associated with neuronal loss (Liao et al. 2012). The hippocampus was selected as BDNF has been previously shown to be more susceptible to alcohol-induced effects on neurotrophic factors than the entorhinal (Miller 2004; Miller and Mooney 2004). Furthermore, BDNF is highly concentrated in the hippocampus compared with other

brain regions (Phillips et al. 1990). Tissue processing for ELISA assays was identical to chapter two.

BDNF content was determined using a Millipore ELISA kit (Billerica, MA; product #CYT306) in accordance with the instructions provided. The total protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Samples were run in duplicate for both the ELISA and BCA and absorbance was measured at 450nm or 595nm, respectively, using a DXT880 Multimode Detector plate reader (Beckman Coulter, Brea, CA). Concentrations were calculated using the line of best fit from corresponding standards and are expressed as pg of cytokine/mg of protein.

Statistical Analysis

Prism Version 5.04 (GraphPad Software, Inc. La Jolla, Ca) was used for all statistical analyses. Behavioral intoxication scores were analyzed with a Kruskal Wallis test followed with Dunn's multiple comparison test, but BECs and ethanol dose per day were analyzed using a one-way ANOVA followed by Tukey's post-hoc test if significance was determined by one-way ANOVA. Two-way ANOVAs were used for analysis of [^{3}H]-PK11195 binding, Iba-1+ cell number, and dystrophic microglia number. Post-hoc Bonferroni corrected t-tests were used following two-way ANOVAs if a main effect or significant interaction was found. Planned post-hoc t-tests were chosen as the comparison of interest was mainly the effect of ethanol diet on measured parameters. Entorhinal cortex and each region of the hippocampus were analyzed separately for [^{3}H]-PK11195 binding and Iba-1+ cell number. BDNF concentrations were compared using a two-tailed, unpaired t-test. All data sets were expressed as mean ± standard error of the mean and analyses considered significantly different if $p<0.05$

RESULTS

Animal model data

BECs were similar among all groups despite variations in binge ethanol exposure duration as shown in Table 3.1. The average BEC for all animals was 344.5 ± 10.2 mg/dL and is comparable to what has been shown previously with this model (Marshall et al. 2013; Morris et al. 2010b). However, the Kruskal Wallis, revealed significant a difference in intoxication behavior variance [$H_{(4)}$ =16.67, p=0.0022]. Dunn's post-hoc analysis of behavioral score revealed a significantly lower behavioral score for animals exposed to ethanol for one day versus four. Accordingly, As dose is dependent upon the intoxication behavioral score, the average administered daily ethanol dose was also significantly different as shown using a one-way ANOVA [$F_{(4,28)}$ =13.21, p<0.0001]. Bonferroni post-hoc analysis of the average dose per day revealed that animals with only one day of exposure received significantly more ethanol per day than either the two or four-day exposed rats. Differences in average daily dose and intoxication behavior were expected as variations in the duration of ethanol exposure have been previously shown to affect these parameters (Hayes et al. 2013).

Table 3.1 Experiment Two Animal Model Data

Experiment	Days of Binge Exposure	Intoxication behavior (0–5 scale)	Dose (g/kg/day)	BEC (mg/dl)
Autoradiography	One (n=5)	0.5± 0.1*	13.4 ± 0.4*	304.1 ± 19.0
	Two (n=5)	1.6 ± 0.1	10.3 ± 0.3	384.8 ± 25.9
Immunohistochemistry	Two (n=8)	1.7 ± 0.2	9.9 ± 0.5	300 ± 18.6
	Four (n=7)	2.2 ± 0.2	9.0 ± 1.2	364.8 ± 23.0
ELISA	Four (n=7)	1.8 ± 0.1	9.6 ± 0.4	371.3 ± 10.4

*Table 3.1 No statistical difference in BEC's were observed despite differences in the intoxication behavior and dose per day in animals that only received one day of ethanol exposure. *p<0.05*

Two days of EtOH exposure results in microglial activation

Binding of [^{3}H]-PK11195 after autoradiography was only measured after one and two days of ethanol exposure as it has been previously shown that [^{3}H]-PK11195 is increased after four days of ethanol exposure (Marshall et al. 2013). No difference was apparent after either one or two days of exposure in binding of the radioligand [^{3}H]-PK11195 in the CA1, DG, or entorhinal cortex compared with controls (Figure 3.1); however, a main effect of diet was observed in the binding of [^{3}H]-PK11195 in the CA2/3 region of the hippocampus [$F_{(1,16)}$ =9.43, p=0.0069]. No significant differences were seen in CA2/3 after one day of exposure, but post-hoc t-tests indicated a significant binding increase of approximate 20% after two days of ethanol exposure [$t_{(8)}$ =4.88, p=0.0018].

Ethanol decreases the number of microglia

The total number of microglia was decreased across multiple regions of the hippocampus of ethanol treated animals compared with controls after both two and four days of ethanol exposure (Figure 3.2). Two-way ANOVAs indicated a significant main effect of diet in the CA1 [$F_{(1,26)}$ =24.49 p<0.0001], CA2/3 [$F_{(1,26)}$ =16.38 p=0.0004], DG [$F_{(1,23)}$ =43.03 p<0.0001], entorhinal cortex [$F_{(1,26)}$ =4.64 p=0.0406], and a significant interaction between diet and time in the CA2/3 [$F_{(1,26)}$ =5.98 p=0.0216]. Planned post-hoc t-tests indicated a significant decrease in microglia number after ethanol exposure in all regions of the hippocampus and the entorhinal cortex after four days of ethanol exposure: CA1 [$t_{(12)}$ =3.18, p=0.0158], CA2/3 [$t_{(12)}$ =3.41, p=0.0104], DG [$t_{(12)}$ =4.70, p=0.0010], and entorhinal cortex [$t_{(12)}$ =2.83, p=0.0302]. However, only microglia number within the CA1 [$t_{(14)}$ =4.79, p=0.0006] and DG [$t_{(14)}$ =4.53, p=0.0010] were decreased following two days of ethanol exposure (Figure 3.2).

Figure 3.1 Increased [^{3}H]-PK-11195 following EtOH Exposure

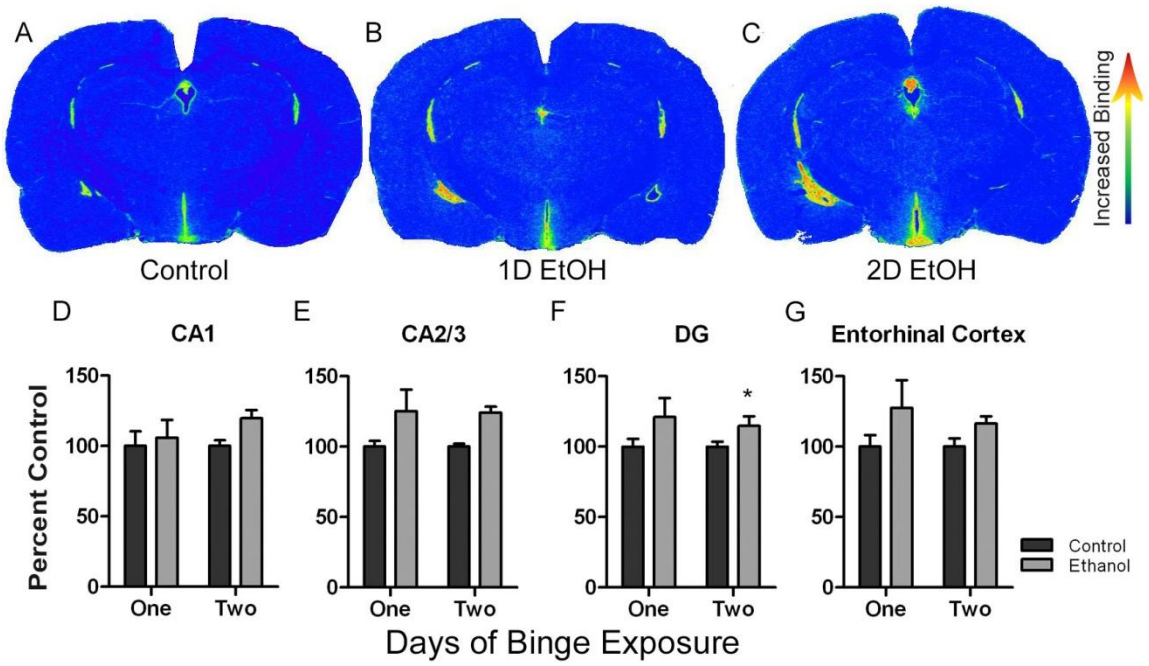


*Figure 3.1. Representative false color autoradiographs depicting [^{3}H]-PK-11195 binding are shown for (A) controls (n = 5; black bars) as well as ethanol (grey bars) after (B) one day of exposure (n=5) or (C) two (n = 6). The legend in the top right corner shows how the false color reflects the intensity of binding. Quantitative analysis of the extent of binding are graphed for the (D) CA1, (E) CA2/3, (F) DG, and (G) entorhinal cortex. An increase in binding was seen after two days of exposure in both the CA2/3 region as well as the DG. *p < 0.05.*

Figure 3.2 Decrease in microglia number during intoxication

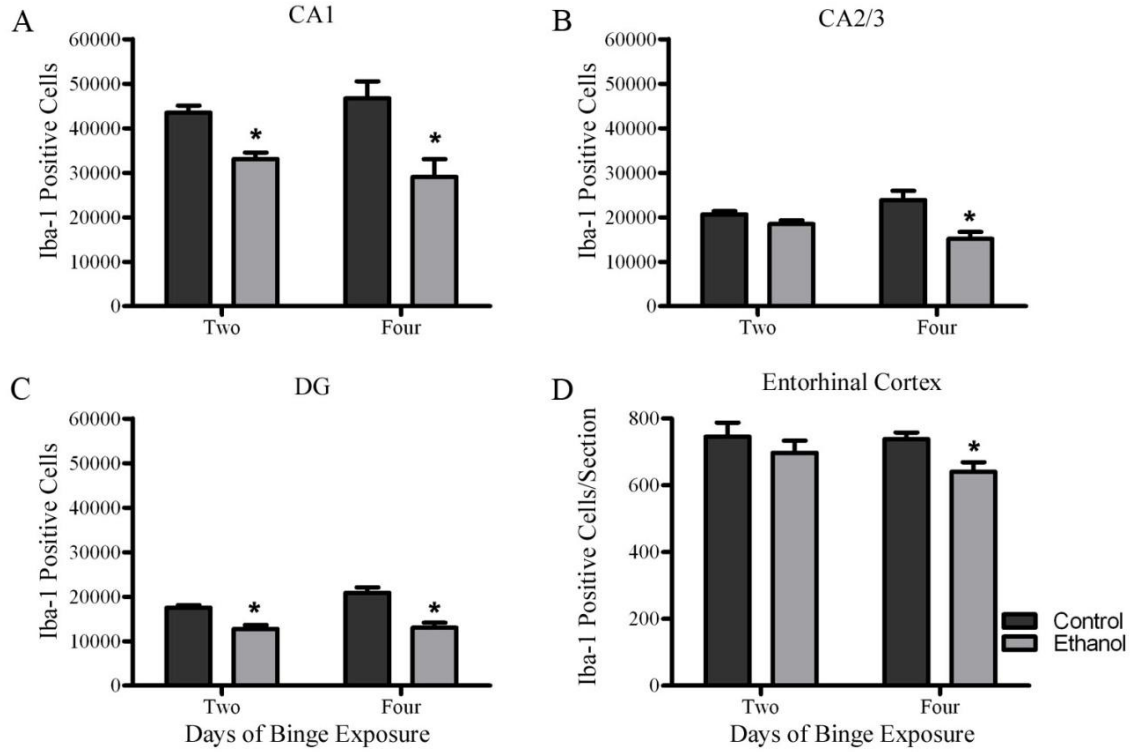


*Figure 3.2. Stereological estimates indicate a decrease in the number of microglia in ethanol treated animals (n = 7; grey bars) compared with control (n = 7; black bars) after four days of exposure in the (A) CA1 (B) CA2/CA3, and (C) DG. Automated cell counts within also indicated a decrease in microglia number in the (D) entorhinal cortex. This decrease can be seen after two days of exposure in the (A) CA1 and (C) DG in ethanols (n = 8) compared with controls (n = 8). *p<0.05.*

Microglia with the unexpected dystrophic morphology were observed in the DG molecular. These oddly shaped cells were quantified using profile counts. Dystrophic microglia, which have been shown to be associated with microglial cell death, were increased following both two and four days of ethanol exposure in the molecular layer of the DG (Figure 3.3). Two-way ANOVAs indicated a significant main effect of diet $F_{(1,25)}$ =16.46 p=0.0004] and time $F_{(1,25)}$ =8.91 p=0.0063]. Importantly, planned post-hoc t-tests

indicated a significant increase in dystrophic microglia after ethanol exposure after two [$t_{(13)}$ =3.37, p=0.0102] and four [$t_{(12)}$ =2.67, p=0.0406] days of exposure.

Figure 3.3 Increase in dystrophic microglia during intoxication

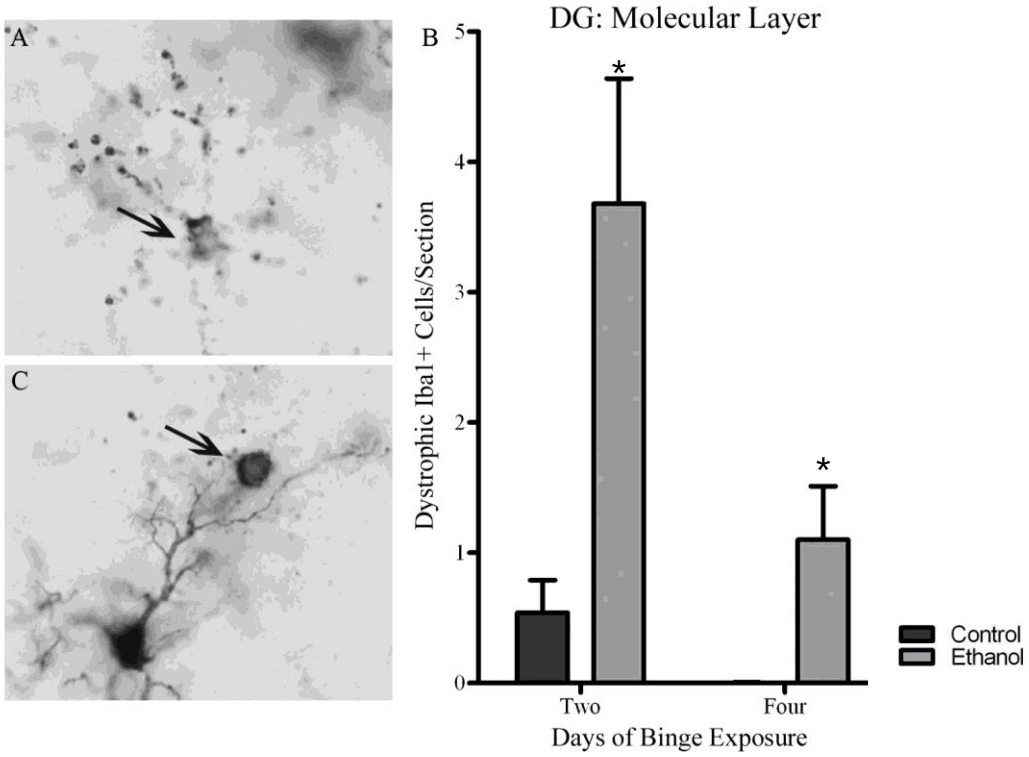


*Figure 3.3. Dystrophic microglia in (A,C) ethanol treated animals are indicated by arrows in representative images. Panel A depict the atypical morphology associated with dystrophic microglia. The arrow points directly at the fragmented cell body with the beaded process. Panel C shows the juxtaposition of a dystrophic microglia to a resting, quiescent cell with typical morphology. Profile counts indicate an increase in the number of (B) dystrophic microglia in ethanol treated animals (n = 7) compared with controls after two (n=8) and four days (n=7) of exposure in the molecular layer of the DG. * p<0.05*

Neurotrophic Factor Decreased by Ethanol Exposure

The loss of microglia was accompanied by a decrease in the concentration of BDNF after four days of ethanol exposure in the hippocampus (Figure 3.4) according to t-test [$t_{(10)}$ =4.22, p=0.0018].

Figure 3.4 Decreased in BDNF following 4 Days of Ethanol Exposure

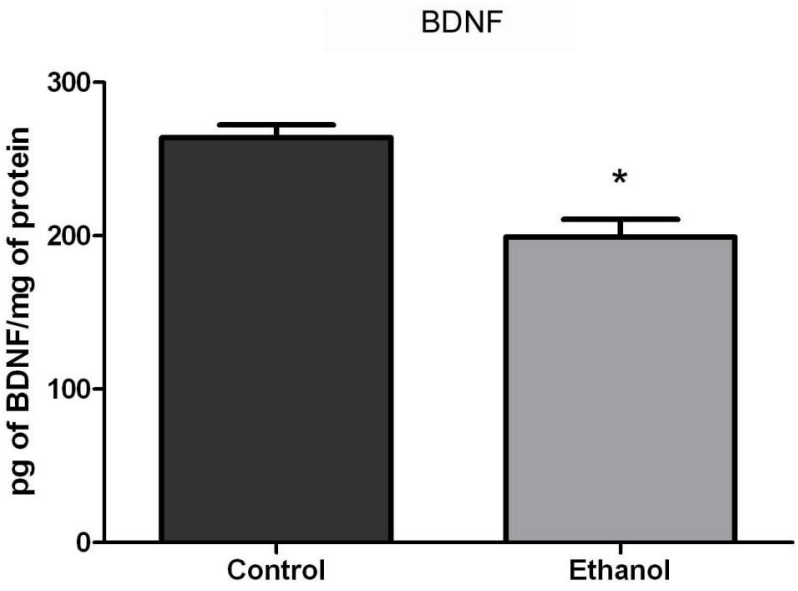


*Figure 3.4 Concentrations of BDNF were determined by ELISA in the hippocampus. There was a 31% decrease in BDNF in ethanol treated animals (n = 7; grey bar) compared with controls (n = 5; black bars). *p < 0.05.*

DISCUSSION

Neuroinflammation has been suggested as source of alcohol-induced damage, but the model of alcohol-induced neurodegeneration used in this report induces low-grade, anti-inflammatory microglial activation that is subsequent to neurodegeneration (Marshall et al. 2013; McClain et al. 2011). This report shows that activation occurs after just two days of ethanol exposure as evidenced by the upregulation of the sensitive microglial activation marker, [^{3}H]-PK11195. Although this change is slight (20%)

compared to four days (250%) of exposure (Marshall et al. 2013), it is still significant. This one and two day exposure model has recently been shown to cause neurodegeneration in both the hippocampus and the entorhinal cortex (Hayes et al. 2013). Whereas neurodegeneration begins after just a single day exposure of ethanol (Hayes et al. 2013), we have shown here that the microglial response is detectable after two days of exposure. The small increase in activation as well as the chronological order of activation and neuronal damage suggests that microglia activation is a response to the initial neurodegeneration induced by alcohol exposure as has been previously proposed (Marshall et al. 2013). Because four days of ethanol exposure does not elicit classical signs of activation like phagocytosis or expression of MHC-II (Marshall et al. 2013), it can be inferred that upregulation of [^{3}H]-PK11195 after two days causes microglia to be partially or alternative activation as well, albeit not specifically characterized in this report. This study is the first *in vivo* study to show that such an acute exposure causes microglial activation, but the evidence concurs with *in vitro* studies showing activation with acute exposure (Bell-Temin et al. 2013).

One criticism of densitometric analysis is the inability to determine if measured differences are due to an increase in numbers of cell expressing the ligand or a change in the protein expression profile. Therefore, the number of microglia was quantified by stereology (hippocampus) or automated cell counts (entorhinal cortex). Both two and four days of binge ethanol exposure caused a decrease in the number of Iba-1+ cells. This finding concurs with human studies that showed a reduction in the number of microglia in the hippocampus of human alcoholics (Korbo 1999) but not with others that showed increases in other brain regions (He and Crews 2008). The decrease in the number of microglia observed indicates that the increases in [^{3}H]-PK11195 reported after

two days of ethanol exposure here and after four days of exposure in chapter two are due to increased activation of microglia.

The reduction in microglia found in regions with alcohol-induced neurodegeneration is surprising because the normal function of microglia is to migrate to areas of damage (Noda and Suzumura 2012). Upon activation, microglia can proliferate and begin to secrete cytokines that attract other microglia. A reduction in microglial seen in both the hippocampus and entorhinal cortex may be indicative of dysfunction as microglial migration to areas of damage is a key component of their function (Damani et al. 2010; Tremblay et al. 2013), but the methods used herein cannot directly contribute the reduction in number to a problem with motility as microglia may directly cause glial damage (Korbo 1999). However, a loss of microglia has been shown to exacerbate damage and represents an alternative mechanism by which alcohol-induced microglia activity may contribute to neurodegeneration (Streit et al. 2009; Wainwright et al. 2009).

The idea that microglial dysfunction can contribute to neurodegeneration is a fairly recent concept (Streit et al. 2009; Streit and Xue 2009). While chronic, over-activation of microglia has repeatedly been proposed as a mechanism for neurodegeneration in various disease states (Lull and Block 2010). The vast effects of microglia mean that the loss of the homeostatic and recovery functions afforded by microglia can also lead to neurodegeneration (Streit et al. 2009). The pathways that lead to neurodegeneration caused by a loss of homeostatic mechanisms afforded by microglia has not been as widely studied as the chronic over-activation pathways, but in models of Alzheimer's Disease and aging has been characterized by either microglial motility impairment (Damani et al. 2010) or the appearance of dystrophic or senescent microglia (Streit et al. 2004b).

Concurrent with the loss of microglia is the increase in the appearance of dystrophic microglia in the molecular layer of the DG. Increased dystrophic, also known as senescent, microglia have been proposed as a mechanism of neurodegeneration and dementia in aging studies as microglia have a distinct role in neurotrophic support both in pathological and nonpathological conditions (Streit et al. 2009). For example, following neuronal injury the brain may recover by increasing neurogenesis (Nixon and Morris 2008). Increased senescent microglia may disrupt this recovery mechanism as microglia are involved at various levels within reactive neurogenesis including proliferation (Morgan et al. 2004), differentiation (Cacci et al. 2008), and neuronal survival through secretion of neurotrophic factors (Kohman and Rhodes 2013; Nakajima et al. 2001; Yoneyama et al. 2011). Alternatively, microglia can act to remove cellular debris from degenerating cells, which also aids in the recovery from brain damage (Czeh et al. 2011; Tremblay et al. 2013). Even in quiescence without any pathological condition, microglia act as alarm systems constantly surveying the neuronal environment and using their ramified branch projections to probe for any abnormalities in the parenchyma (Nimmerjahn et al. 2005). The loss of any of these microglial properties of mechanisms makes the increase in the number of dystrophic microglia coupled with a loss in the number of a critical concern in normal neuronal function and/or response to damage.

Given that the microglial response seen after binge ethanol exposure appears to be partial activation which is associated with neurotrophic support, the decreases in BDNF seen herein may potentially be due to the dysfunction of microglia. The normal response of microglia following neuronal injury and activation is to upregulate production of BDNF (Miwa et al. 1997; Nakajima et al. 2001). However, because BDNF is a secreted protein produced by astrocytes, neurons, and endothelial cells (Bejot et al.

2011), this study cannot directly tie the decrease in BDNF solely to microglial function. Alcohol-induced deficits in microglia number and increased senescence may be partially responsible for decreased BDNF concentrations. Decreased BDNF during intoxication found here agrees with previous reports on the effect of ethanol on hippocampal BDNF in vapor inhalation models of an AUD (Tapia-Arancibia et al. 2001) as well as the decreased levels seen in the serum of human alcoholics (Davis 2008; Joe et al. 2007). BDNF is associated with the survival of neurons following proliferation (Lee and Son 2009; Lee et al. 2002; Loeliger et al. 2008). Intriguingly, the deficits in BDNF occur simultaneously with the reduction of cell survival of newly proliferated cells previously reported in this model (Nixon and Crews 2002). Together reduced newborn cell survival and BDNF levels further alludes to a potential role of microglia dysfunction within alcohol-induced neurodegeneration.

These results altogether show that microglia become activated early within the Majchrowicz AUD model, but likely in response to alcohol-induced neurodegeneration given the low-nature of activation as well as the chronological order of activation and neuronal cell death. This early activation, however, is concurrent with signs of microglial loss and the appearance of dystrophic microglia. The loss of the neuroprotective function of microglia during intoxication may serve as a potential source of neurodegeneration by inhibiting recovery. A direct relationship between the effects of alcohol on microglia and neurodegeneration is still yet to be elucidated, but interpretations of the data included herein provide an alternative view on how microglia may be involved with alcohol-induced neurodegeneration. It has become increasingly evident that multiple neurobiological systems are involved with alcohol brain damage (Crews and Nixon 2009; Kruman et al. 2012), but the loss of neuroprotection/homeostatic functions of microglia could further cause deterioration in an already vulnerable system. Rescuing microglia

loss during intoxication through pharmacological agents may provide a valid, novel therapeutic option for reduction of alcohol-induced neurodegeneration.

Chapter 4: Ethanol can potentiate the primed microglial response in an alcohol-induced neurodegeneration model

INTRODUCTION

Chronic, excessive consumption of alcohol can result in neurodegeneration (Crews and Nixon 2009; Pfefferbaum et al. 1992; Zahr et al. 2011). This neurodegeneration and its associated cognitive deficits are thought to play a role in the development of an AUD (Crews and Boettiger 2009; Koob and Le Moal 1997). Understanding how this neurodegeneration occurs may provide a therapeutic target for the treatment of AUDs. Recently, it has been proposed that microglial activation is a potential mechanism that causes neurodegeneration in individuals who suffer from an AUD (Crews et al. 2011; Qin et al. 2013). Evidence in the brain of human alcoholics suggest that excessive alcohol consumption leads to microglial activation (He and Crews 2008; He et al. 2005), but whether this activation is causative in alcohol-induced neurodegeneration is currently debatable. This debate is due in part to variations in the level of microglial activation among AUD models with brain damage (Qin et al. 2008; Zahr et al. 2010a). In neurodegenerative diseases where microglial activation has been shown to be a driving mechanism in neuronal cell loss (Block and Hong 2005; Brown and Neher 2010; Smith et al. 2012), microglia are fully activated over a long period of time secreting proinflammatory factors and undergoing uncontrolled phagocytosis (Brown and Neher 2010; Fricker et al. 2012; Streit et al. 2004a). While some AUD models indicate proinflammatory microglia (Qin and Crews 2012a; Qin et al. 2008; Ward et al. 2009a), others report a more low-grade level of activation that may be beneficial or neuroprotective (Marshall et al. 2013; McClain et al. 2011; Zahr et al. 2010a). Two reasons that have been proposed for the discrepancies across models in microglial activation are (1) intermittent versus sustained intoxication within a model as well as (2)

the presence or lack of systemic immune influences (Marshall et al. 2013; Zahr et al. 2010a).

The Majchrowicz AUD model is a model with high BECs that likely do not fall below intoxication (Morris et al. 2010b). This model also has an intact BBB that prevents systemic immune influences on microglia (Marshall et al. 2013). Likewise, this model has recently been reported as inducing low-grade microglial activation rather than proinflammatory microglia (McClain et al. 2011). Although the Majchrowicz model produced evidence of low-grade activation, microglial activation and increases in cell number were shown to be persistent and lasted at least a month after the last dose of ethanol (Marshall et al. 2013; Obernier et al. 2002b). Persisting, low-level activation can alter the neuroimmune system such that future neuroimmunomodulators have an exacerbated or potentiated response (Bilbo and Schwarz 2009; Bland et al. 2010). A potentiated response can change microglial from a low-grade, neurotrophic state to the more classical activation phenotype associated with neuronal damage (Lewis 2012; Norden and Godbout 2013). This phenomenon is known as microglial priming and is an alternative explanation for the discrepancy in the level of activation seen between AUD models (Dilger and Johnson 2008; Marshall et al. 2013; McClain et al. 2011; Norden and Godbout 2013). Alcohol exposure may prime microglia so that subsequent insulting exposures or intermittent bingeing act as secondary neuroimmune modulators that then alter the microglial response.

Ethanol's ability to act as a priming agent and exacerbate the neuroimmune response of stimuli that mimic systemic infection has already been shown (Qin and Crews 2012a; Qin et al. 2008; Qin et al. 2013). The current study determines whether ethanol exposure alone can act as both the priming agent and secondary neuroimmune modulator by giving a secondary "hit" or binge exposure. Individuals suffering from an

AUD binge drink in a more episodic fashion and show signs of alcohol-induced neurodegeneration in the absence of systemic influences (Epstein et al. 2004; Hunt 1993; Paradis et al. 2009). This drinking pattern makes understanding the nature of secondary exposure vital to a full view of the effects of microglia in neurodegeneration. If ethanol alone potentiates the microglial response, it could be indicative of a feed-forward/back process such that repeated exposure causes a loop of activation and elicits a microglial response that is more proinflammatory and damaging in nature (Crews et al. 2011).

The current experiments examine how repeated ethanol exposure affects microglia. Specifically, this study uses both functional and morphological indices to determine the level of microglial activation in the hippocampus and entorhinal cortex, regions damaged in this binge paradigm (Collins et al. 1996; Kelso et al. 2011; Obernier 2002). The level of activation was assessed to determine if ethanol alone could potentiate the microglial response and switch the low-grade phenotype elicited by the Majchrowicz model to a more classical activation state with repeated exposure.

MATERIALS AND METHODS

Alcohol Administration Model

A total of 33 adult male Sprague-Dawley rats (Table 4.1; Charles River Laboratories, Raleigh, NC) were used in these experiments. Procedures performed were approved by the University of Kentucky Institutional Animal Care and Use Committee and were within the Guidelines for the Care and Use of Laboratory Animals (NRC 1996). Animals were 275-300g upon arrival and were pair-housed in a University of Kentucky AALAC accredited vivarium with a 12h light:dark cycle. Rats were allowed to acclimate to the vivarium for two days followed by three days of handling before any

experimentation. During this acclimation period, animals had *ad libitum* food and water access.

Rats were divided into four groups of comparable weights. As shown in Table 4.1, three of the four groups were subjected to a modified version of the Majchrowicz AUD model. This model has previously been described in chapter two, and the binge methods used in these experiments were identical. Animals underwent the four-day Majchrowicz AUD paradigm with intragastric gavage and were then given seven days of recovery with *ad libitum* access to food and water. A seven day recovery period was chosen because it has previously been shown that microglial activation is elevated to consistent levels for a week after ethanol exposure (Marshall et al. 2013). Furthermore, seven days allowed animals to regain body mass loss during intubation procedures. Following the recovery period the Majchrowicz binge model was repeated giving either ethanol or control diet (see Table 4.1 for details). The entire treatment period was fifteen days, only eight of which included intragastric gavage exposure. A separate group had *ad libitum* access throughout all periods. For all groups, body weights were assessed daily during the binge procedures. The percent weight difference was calculated comparing weights at the start and end of the 15-day treatment period.

To determine BECs, tail blood was collected ninety minutes after the seventh session of ethanol dosing during the first binge exposure (Binge 1) and at euthanization (Binge 2) within hours of the final dose. Samples were centrifuged for 5 min at 1800g to separate plasma from red blood cells and immediately stored at -20˚C to avoid sample degradation. BECs were determined using 5µL of supernatant serum an AM1 Alcohol Analyser (Analox, London, UK). Each sample was run in triplicates that were calibrated against a 300mg/dL external standard. The average of these runs was calculated and expressed in mg/dL.

Table 4.1 Treatment Summary

Group	Binge1 (4 Days)	Recovery (7 Days)	Binge 2 (4 Days)
Ad libitum (n=4)	N/A	*Ad libitum* food and water access	N/A
Con/Con (n=10)	Control Diet		Control Diet
Con/EtOH (n=11)	Control Diet		Ethanol Diet
EtOH/EtOH (n=8)	Ethanol Diet		Ethanol Diet

Table 4.1. Animals were divided into four groups. The first group, ad libitum, was allowed access to food and water throughout all treatment periods. All other groups had four days of intragastric gavage 3 times a day, a seven-day recovery period, and then a second treatment period of intragastric gavage. Groups are labeled based on their treatment such that the Con/Con and EtOH/EtOH group received either control (Con) or ethanol (EtOH) diet, respectively, during both treatment periods, but the Con/EtOH group of animals first received control diet and then EtOH diet in the second treatment period.

Figure 4.1 A Timeline of Animal Treatment

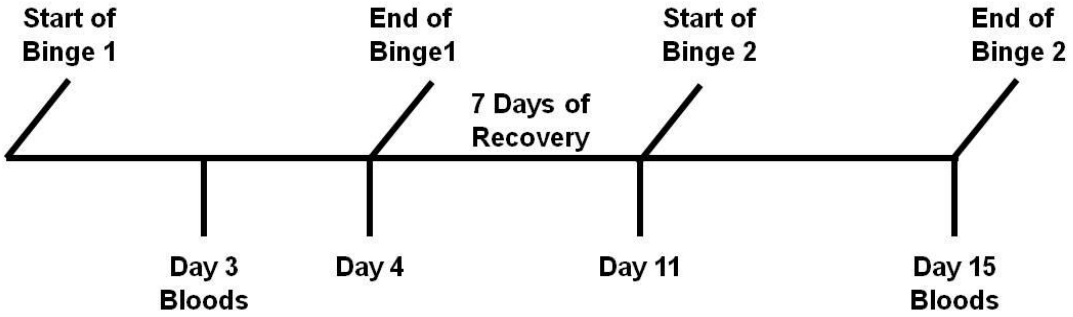


Figure 4.1 A timeline of the binge treatment, recovery, and blood collections that animals underwent.

Rats were euthanized within hours of their final treatment by rapid decapitation. Brains were extracted and dissected into two hemispheres on ice. The left hemisphere was fixed by immersion in 4% paraformaldehyde in PB (pH=7.4) and used in immunohistochemistry experiments. The right hemisphere, however, was further dissected such that the hippocampus and entorhinal cortex were removed. Extracted regions were snap frozen on dry ice for use in cytokine analysis using ELISA.

Immunohistochemistry

The left hemisphere was sectioned and underwent immunohistochemical processing identical to the treatment of brains described in previous reports as well as in chapter two (Marshall et al. 2013). However due to changes in how the brain was processed (i.e. no perfusions), tissue was incubated in primary antibodies at 4˚C as follows: mouse anti-OX-6 (1:500, Serotec, Raleigh, NC), mouse anti-ED-1 (1:500; Serotec), rabbit anti-Iba-1 (1:1000, Wako, Richmond, VA), or mouse anti-OX-42 (1:1000; Serotec). Primaries were chosen for their specificity for microglia phenotypes as described in chapter 2 (Table 2.1). Methods for the application of secondary antibody (biotinylated horse anti-mouse, rat adsorbed, or biotinylated goat anti-rabbit, Vector Laboratories, Burlingame, CA), avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) and chromagen, DAB (Polysciences, Warrington, PA), were identical for all primary antibodies and followed previously published methods as well as in chapter two (Marshall et al. 2013; McClain et al. 2011). Following the final wash, all stained sections were mounted onto glass slides and dried before being coverslipped with Cytoseal® (Stephens Scientific, Wayne, NJ).

Quantification

Slides were coded to ensure the experimenter was blinded to treatment conditions during quantification. OX-42 quantification and qualitative assessments of ED-1, and OX-6 were identical to methods described in chapter two and previously reported (Marshall et al. 2013). OX-42 results were averaged and expressed as percent control.

Iba-1+ cells were quantified in the entorhinal cortex by an automated counting system, Image Pro Plus 6.3 (Media Cybernetics, Rockville, MD) as previously described; however, images containing the entire entorhinal cortex were collected at 6.4x between

using SPOT Advanced™ (SPOT Imaging Solutions, Sterling Heights, MI). The number of cells per section was averaged and expressed as Iba-1+ cells/section.

Iba-1+ cells within the subregions of the hippocampus were estimated by unbiased stereological methods as described in previous chapters and were identical to previously published reports (Marshall et al. 2013). For all stereological quantifications, coefficient of error ranged from 0.011 to 0.039 and averaged 0.023 ± 0.001

Enzyme Linked Immunosorbent Assay

Tissue collected for ELISA studies was manually homogenized in an ice-cold lysis buffer (1mL of buffer/50mg of tissue; pH=7.4). The buffer and homogenate preparation was consistent with other reports as detailed in previous chapters (Marshall et al. 2013; Rabuffetti et al. 2000). Cytokine protein content in the hippocampus and entorhinal cortex was determined with an ELISA kit according to the manufacturer's instructions for TNF-α (Invitrogen product #KRC3011C, Camarillo, CA) and IL-10(Invitrogen product #KRC0101). These two cytokines were used to understand the pro or anti-inflammatory nature of microglia, respectively. However, BDNF was only measured in the hippocampus (Billerica, MA; product #CYT306). Testing only one hemisphere resulted in a limited sample so only these cytokines or growth factors were assessed. All samples and standards were run in duplicate. Absorbance was measured at 450nm for ELISA or at 595nm for the BCA assay on a DXT880 Multimode Detector plate reader (Beckman Coulter, Brea, CA). The cytokine protein concentration was divided by the total protein concentration obtained in the BCA assay to correct for differences in tissue volume and reported as pg of cytokine/ mg of protein.

Statistical Analyses

The data were analyzed and graphed using Prism Version 5.04 (GraphPad Software, Inc. La Jolla, Ca) and reported as the mean ± standard error of the mean.

Analyses were considered significantly different if $p<0.05$. Behavioral scores were analyzed with a Kruskal Wallis test. All other parametric analyses were analyzed using a one-way ANOVA with post-hoc Tukey's multiple comparison tests to compare between groups if an effect of treatment was observed. Where appropriate, each region of the hippocampus or entorhinal cortex was considered independent and therefore analyzed separately. To address the potential additive effects of ethanol, correlation analyses were conducted looking at the relationship of microglial markers of activation and the animal model data. Correlation analyses also were conducted comparing cytokine concentration to immunohistochemical measures of microglia since cytokines measurements and immunohistochemical quantifications were done within the same animal. Analysis of immunohistochemical results and ELISA data also allowed for a better interpretation of the source of cytokines. Correlations were only run within the Con/EtOH or EtOH/EtOH group if post-hoc analyses showed a significant difference to control groups. Spearman analyses were used for intoxication behavior scores as they are nonparametric, while Pearson's analyses were used for all other factors (i.e. percent weight difference, BEC, etc.).

RESULTS

Animal Treatment Data

Although the Vanilla Ensure Plus® diet is considered nutritionally complete, percent difference in weight was calculated to assess whether restricted caloric intake affected microglia activation (Loncarevic-Vasiljkovic et al. 2012; Tu et al. 2012). One-way ANOVA indicated that treatment differentially affected percent weight change [$F_{(2,24)}=4.235$, $p=0.0266$] (Table 4.2). Tukey's post-hoc analysis showed that the percent weight change differed between all the groups that had intragastric gavage (Con/Con,

Con/EtOH, and EtOH/EtOH) compared with the *ad libitum* group. Importantly, post-hoc tests showed no difference between the weight change in the Con/EtOH and EtOH/EtOH groups. For the analysis of binge subject data, each binge period per group was considered independently such that data for animals that received ethanol twice were analyzed as separate entities. For example, BECs from Binge 1 and 2 for the EtOH/EtOH were analyzed as separate data points. No differences were detected in either intoxication score (grand mean=1.6 ± 0.1) or in BECs (grand mean=399.8 ± 12.4 mg/dL) for any of the treatment periods or groups (Table 4.3). However, one-way ANOVA analysis revealed differences in the average dose per day [$F_{(2,24)}$ =4.235, p=0.0266]. A post-hoc Tukey's test indicated a significant difference in the dose per day during Binge 2 comparing the EtOH/EtOH and Con/EtOH rats (Table 4.3).

Table 4.2 Percent Body Weight Change

Group	Percent Weight Change
Ad libitum (n=4)	+25.2% ± 1.7
Con/Con (n=10)	+1.0% ± 1.4$^{\$}$
Con/EtOH (n=11)	-6.6% ± 2.1*
EtOH/EtOH (n=8)	-8.7% ± 1.7*

*Table 4.2 The percent weight change was calculated for each treatment group. *p < 0.05 compared to ad libitum and Con/Con group; $^{\$}$p<0.05 compared to ad libitum group only.*

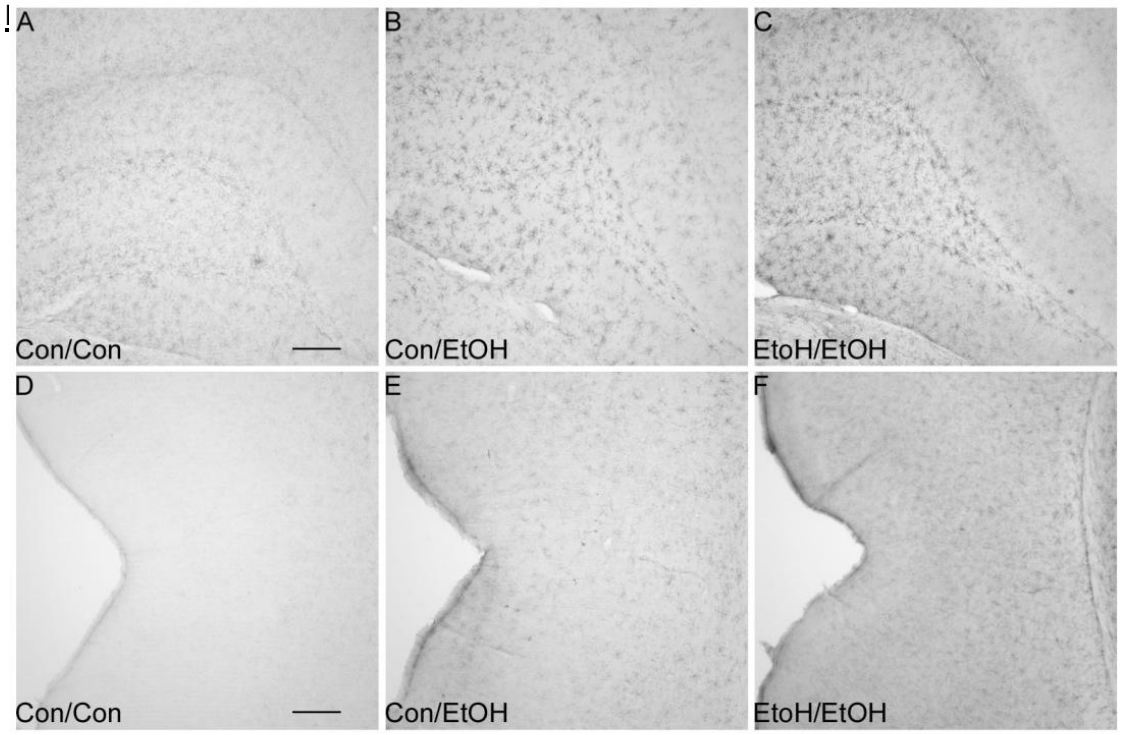
Table 4.3 Experiment Three Animal Model Data

Group	Intoxication behavior (0–5 scale)	Dose Per Day (g/kg/day)	BEC (mg/dl)
Con/EtOH	1.8 ± 0.1	9.6 ± 0.2	422.2 ± 21.1
EtOH/EtOH Binge 1	1.7 ± 0.1	9.9 ± 0.4	378.7 ± 17.7
EtOH/EtOH Binge 2	1.3 ± 0.2	11.0 ± 0.5$^{\#}$	390.3 ± 24.02

Table 4.3 Measures of various intoxication parameters of the Majchrowicz model are statistically similar between all treatment groups excluding the dose per day given to EtOH/EtOH animals during the second treatment compared with the dose per day given to the Con/EtOH group. Since all other parameters are similar, it is not likely that the dose per day affected any other outcome measures. $^{\#}p < 0.05$ *compared to Con/EtOH.*

OX-42 immunoreactivity increased by EtOH exposure

OX-42 expression was examined to determine whether microglia were further or differentially activated following secondary binge exposure. OX-42 positive cells were apparent in all treatment groups, which is consistent with its constitutive expression in all types of microglia (Akiyama and McGeer 1990)**.** However**,** there was a visibly distinct increase in immunoreactivity in ethanol treated animals and an apparent morphological change indicated by a reduction in the ramification but a thickening of the processes in the ethanol animals compared with the controls (Figure 4.2, A-F). One-way ANOVAs indicated a significant effect of treatment in the CA1 [$F_{(3,29)}$ =16.81, p<0.0001], CA2/3 [$F_{(3,29)}$ =18.34, p<0.0001], and DG [$F_{(3,29)}$ =14.43, p<0.0001] fields, as well as in the entorhinal cortex [$F_{(3,28)}$ =19.01, p<0.0001]. As expected based on the data detailed in chapter two, Post-hoc Tukey's tests indicated a significant increase in OX-42 density in all ethanol treated groups' in all regions compared with the control or *ad libitum* group. Importantly, the EtOH/EtOH group showed greater immunoreactivity than Con/EtOH in all regions except the DG. Moreover, no difference in staining was observed between *ad libitum* animals and the Con/Con group. Correlation analyses of binge model measure with OX-42 immunoreactivity were run within the EtOH/EtOH and Con/EtOH group (Table 4.4).


A
B
C
Con/Con
Con/EtOH
EtoH/EtOH
D
E
F
Con/Con
Con/EtOH
EtoH/EtOH

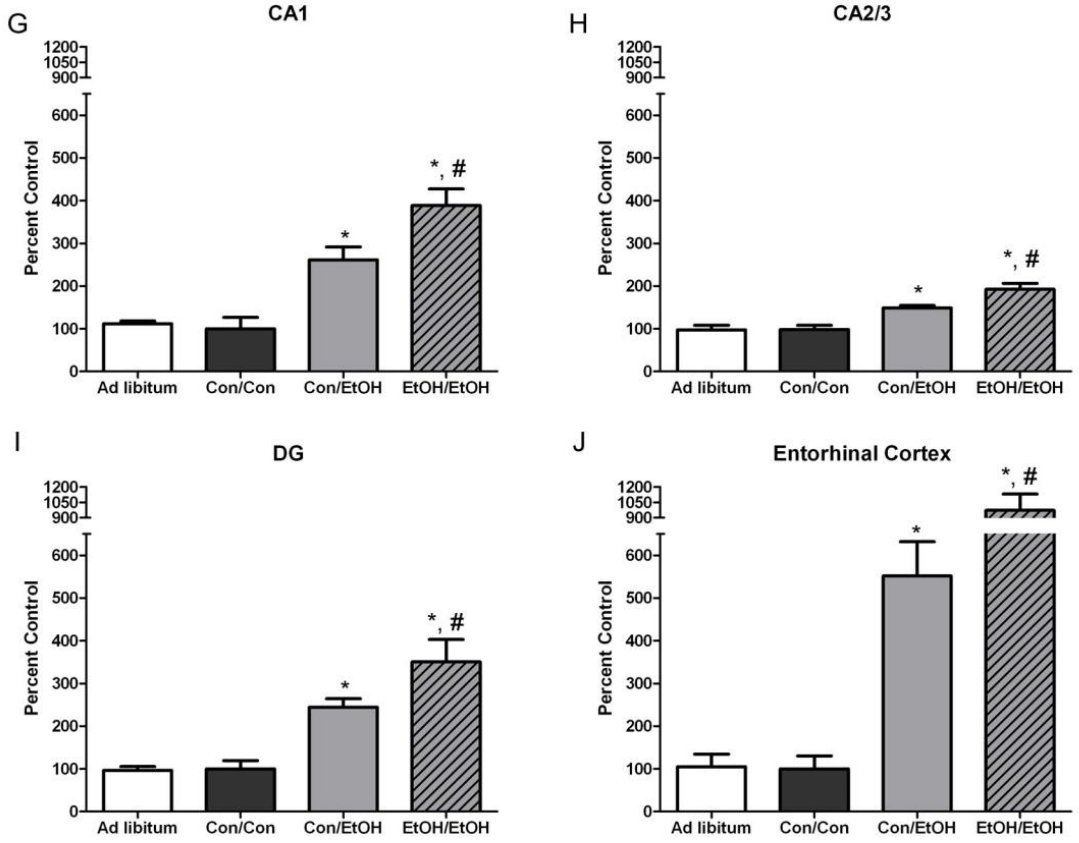

G
CA1
H
CA2/3
I
DG
J
Entorhinal Cortex
Percent Control
1200
1050
900
600
500
400
300
200
100
0
*
*, #
Ad libitum
Con/Con
Con/EtOH
EtOH/EtOH

*Figure 4.2. CD11b is upregulated in the hippocampus and entorhinal cortex as shown in representative photomicrographs of the (A-C) hippocampal DG and the (D-F) entorhinal cortex for (B,E) Con/EtOH (C, F) and EtOH/EtOH group compared to (A, D) controls. Analysis of OX-42 immunoreactivity indicated the EtOH/EtOH group had significantly more staining than the Con/EtOH group in the: (G) CA1, (H), CA2/3, and (I) DG as well as the (J) entorhinal cortex. Scale bars=200 µm. *p < 0.05 compared to ad libitum and Con/Con group; #p< 0.05 compared to Con/EtOH.*

Table 4.4 OX-42 Immunoreactivity Correlation Analyses

Group Region	Parameter	Correlation Coefficient	P-value
EtOH/EtOH-Hippocampus	Intoxication behavior	S=0.523	0.20
	Dose/Day	P= -0.053	0.90
	Total Dose	P= -.0267	0.52
	BEC	P=-0.572	0.13
	Percent Weight Loss	P=0.249	0.55
	Iba-1+ Cells	P= 0.539	0.17
EtOH/EtOH-Entorhinal Cortex	Intoxication behavior	S=0.371	0.36
	Dose/Day	P= -0.456	0.30
	Total Dose	P= -0.575	0.18
	BEC	P=0.032	0.94
	Percent Weight Loss	P=0.319	0.46
Con/EtOH-Hippocampus	Intoxication behavior	S=0.433	0.21
	Dose/Day	P= -0.321	0.37
	BEC	P=0.424	0.22
	Percent Weight Loss	P=-0.222	0.54
Con/EtOH-Entorhinal Cortex	Intoxication behavior	S=0.628	0.06
	Dose/Day	P= -0.488	0.15
	BEC	P=-0.082	0.82
	Percent Weight Loss	P=0.029	0.94

Table 4.4 No significant correlations were found between OX-42 immunoreactivity and animal model data or cell number.

Lack of ED-1 or OX-6 positive cells

The ED-1 antibody was used to recognize phagocytic microglia, whereas OX-6 was used to visualize the upregulation of MHC-II (Graeber and Streit 2009; Raivich et al. 1999a). No groups appeared to have an upregulation of ED-1 (Figure 4.3) or OX-6 (Figure 4.4) positive cells within the hippocampus or entorhinal cortex. There was, however, one EtOH/EtOH treated animal that several OX-6 cells within the more posterior regions of the hippocampus and entorhinal cortex (Figure 4.4 D, H). The animal with increased OX-6 cells was not an outlier for any intoxication parameter

including BEC, intoxication behavior, or ethanol dose per day. Interestingly, the morphology of these cells still appeared to be characteristic of the low grade, partial activation state of microglia. ED-1 and OX-6 positive cells were visible in blood vessels, the hippocampal fissure, and along the meninges in all treatment groups (Figure 4.3; 4.4) similar to that previously reported following binge ethanol exposure (Marshall et al. 2013; Nixon et al. 2008). Thus, repeated exposure of ethanol treatment failed to significantly induce phagocytic-stage microglia or increase MHC-II in the brain parenchyma.

Figure 4.3 Lack of ED-1 Positive Cells

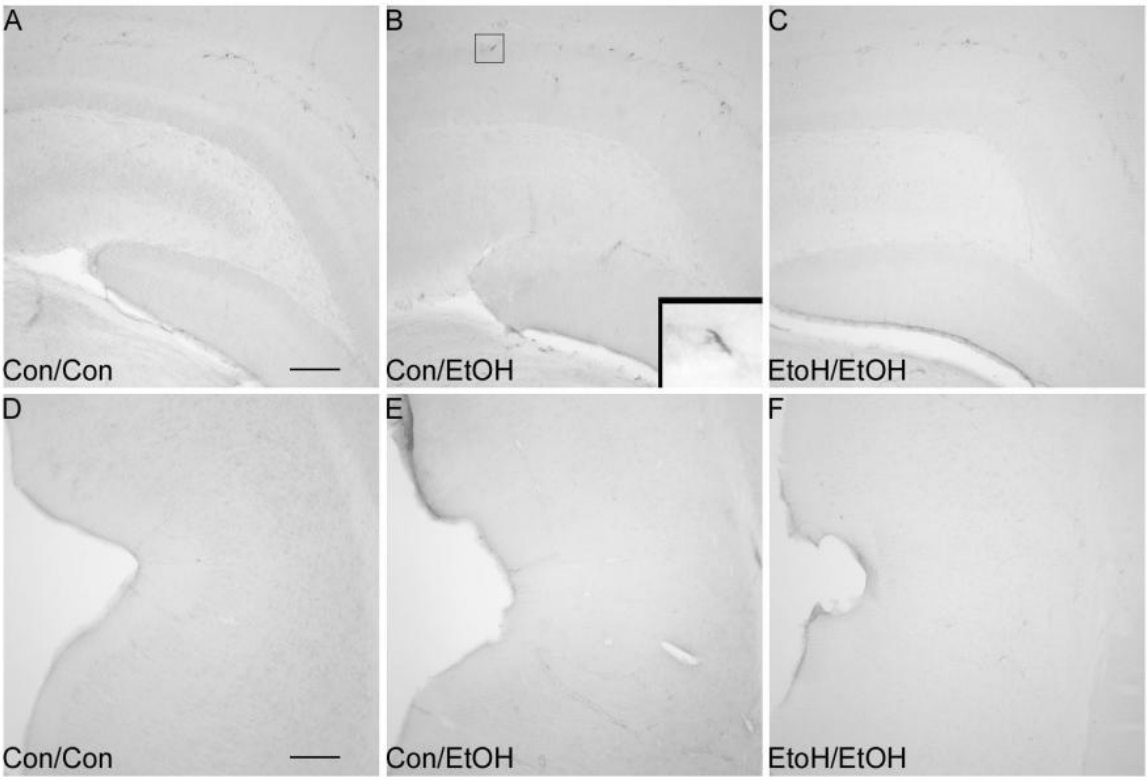


Figure 4.3. *ED-1 was not visible in the parenchyma of the (A–C) hippocampus or (D–F) entorhinal cortex as seen in representative photomicrographs in (A, D) controls, (B,E) Con/EtOH (C, F) or EtOH/EtOH group. ED-1 positive cells could be seen along the blood vessels as shown in the inset of B. Scale bars=200 µm.*

Figure 4.4 Lack of OX-6 Positive Cells

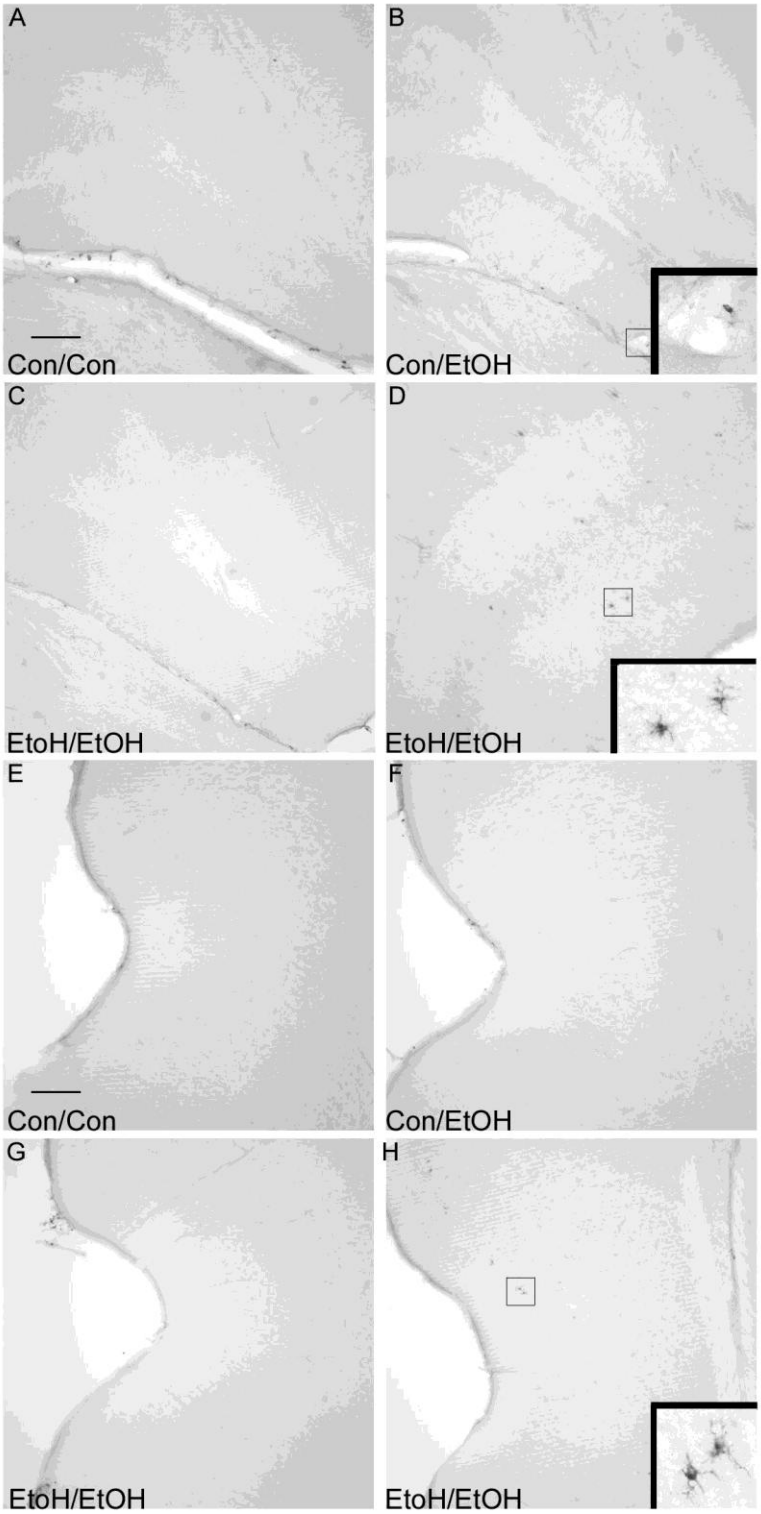


Figure 4.4. No OX-6 positive cells were visualized regardless of treatment in the majority of animals as seen in representative photomicrographs of the (A-C) hippocampus or (E-H) entorhinal cortex in (A, E) controls, (B,F) Con/EtOH (C, G) or EtOH/EtOH group. However, OX-6 positive cells could be seen along blood vessels as shown in the inset of B. One EtOH/EtOH animal showed upregulation of OX-6 in both the (D) hippocampus and (H) entorhinal cortex. Scale bars= 200 µm.

Differential effects of treatment on number of microglia

Stereology and automated cell counts were used to determine whether repeated ethanol exposure affects the number of microglia during exposure (Figure 4.5). One-way ANOVAs indicated a significant effect of treatment in the CA1 [$F_{(3,29)}$ =161.6, p<0.0001], CA2/3 [$F_{(3,29)}$ =17.99, p<0.0001], DG [$F_{(3,29)}$ =69.98, p<0.0001] fields, as well as in entorhinal cortex [$F_{(3,28)}$ =6.78, p=0.0014]. Post-hoc Tukey's tests indicated a significant increase in the number of Iba-1+ cells in all subregions of the hippocampus in the EtOH/EtOH group compared with all other groups. However, in the entorhinal cortex microglia cells in the EtOH/EtOH group were decreased compared to the *ad libitum* and control groups but was similar to the number seen in Con/EtOH treated animals (Figure 4.5). Consistent with data discussed in chapter 3, Tukey's multiple comparison tests showed that Con/EtOH rats had decreased Iba-1+ cells in all regions measured compared to Con/Con and *ad libitum* groups (Figure 4.5). Because the number of microglia can affect immunoreactivity, an analysis of the number of microglia compared with OX-42 immunoreactivity was run, but no significant relationship was determined (Table 4.4)

Figure 4.5 Differential effects of Repeated Exposure on the number of Microglia

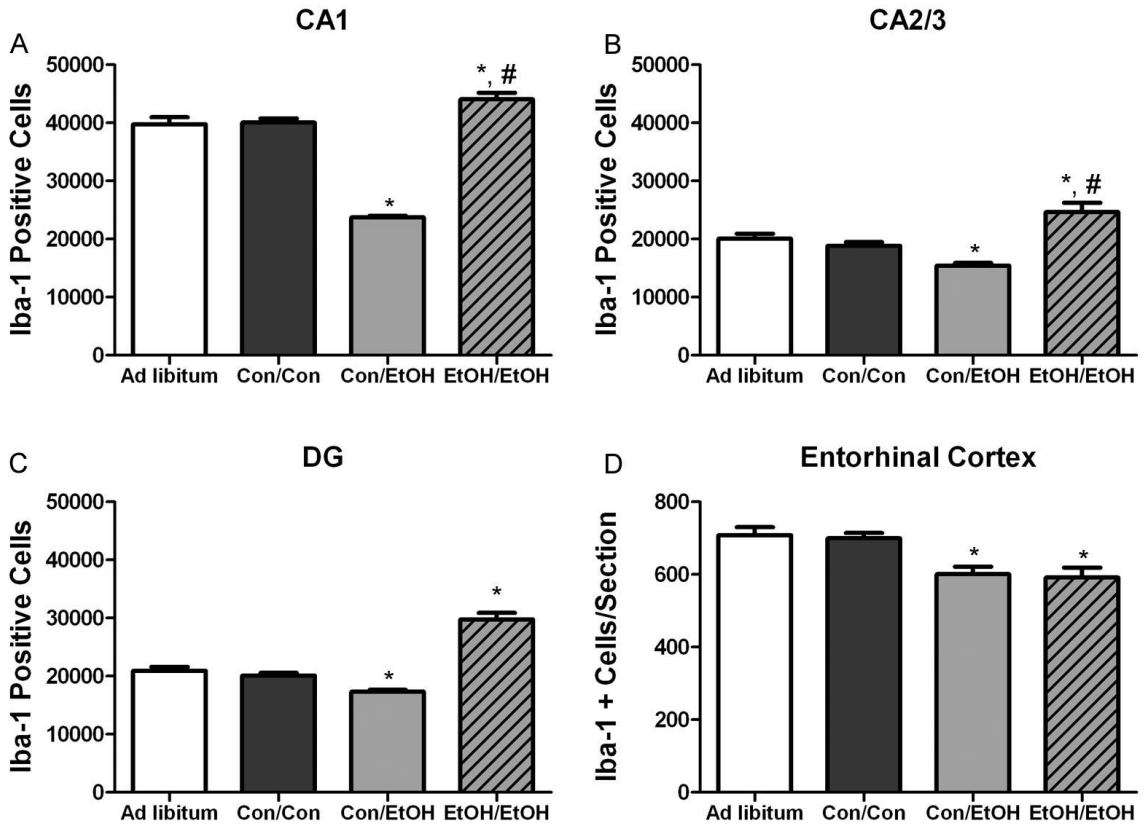


*Figure 4.5. Stereological estimates indicate an increase in the number of microglia in the EtOH/EtOH group in the (A) CA1 (B) CA2/CA3, and (C) DG compared with all other treatment groups. However, the number of microglia in the Con/EtOH group was consistently decreased throughout the hippocampus. In the (D) entorhinal cortex, microglia cells were decreased in both the Con/EtOH and EtOH/EtOH treated groups compared to both the ad libitum and Con/Con groups. *p < 0.05 compared to ad libitum and Con/Con group; # p < 0.05 compared to Con/EtOH.*

Increased TNF-α in EtOH/EtOH group

ELISAs were used to assess the functional state of microglia, specifically the anti-inflammatory cytokine, IL-10, and the proinflammatory cytokine TNF-α. Consistent with results in chapter two, no changes were seen in IL-10 during intoxication in either the hippocampus or the entorhinal cortex in the Con/EtOH or EtOH/EtOH groups (Figure 4.5). However, a one-way ANOVA on TNF-α concentrations indicated a significant effect of treatment in the hippocampus [$F_{(3,28)}$ =4.658, p=0.0092] but not the entorhinal cortex. Post-hoc Tukey's tests indicated a significant increase in TNF-α in the hippocampus in the EtOH/EtOH group compared with all other groups (Figure 4.6). The distribution of TNF-α concentrations observed in the EtOH/EtOH group did not fit a normal distribution and appeared to be bimodal. Correlation analyses of binge parameters as well as immunohistochemical results were run within the EtOH/EtOH group to further probe the bimodal distribution of the TNF-α concentrations within the hippocampus (Table 4.5). BECs correlated with TNF-α concentration [$P_{(8)}$ =0.807, p=.0155] (Table 4.5; Figure 4.7).

Figure 4.6 Increased TNF-α in EtOH/EtOH group

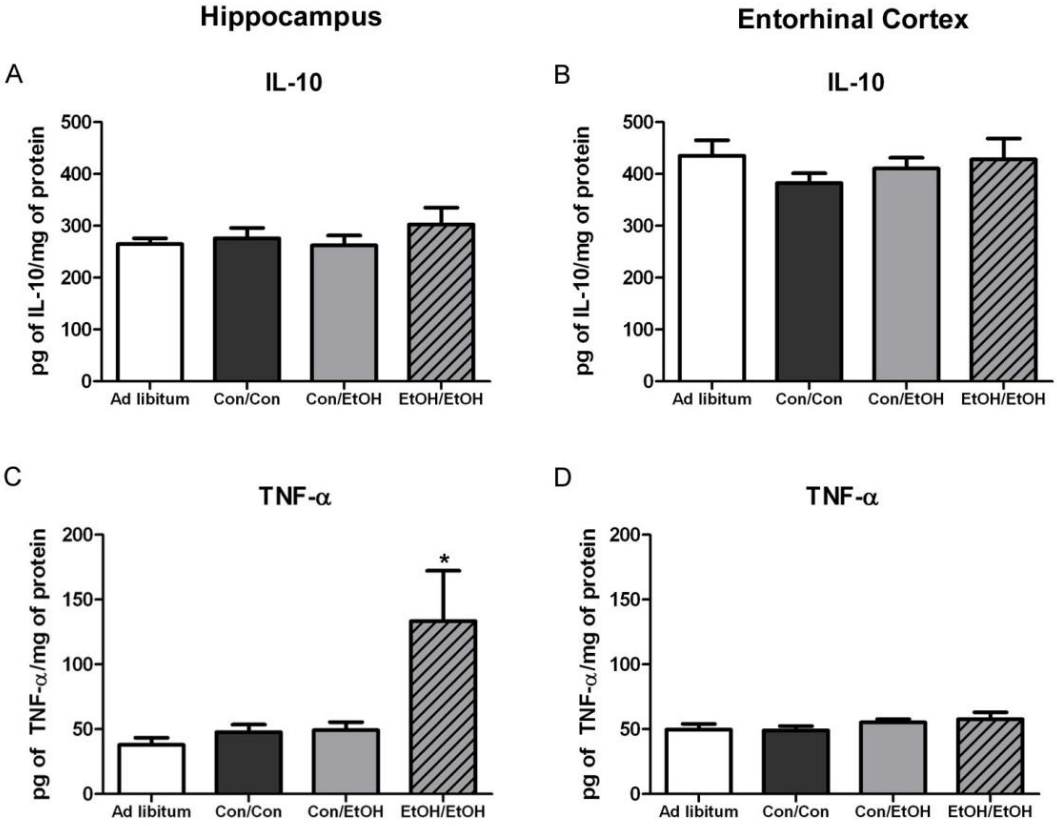


*Figure 4.6. Concentrations of (A, B) IL-10 and (C, D) TNF-α were determined by ELISA in both the hippocampus (A, C) and entorhinal cortex (B, D). No change in IL-10 was measured in either the hippocampus or entorhinal cortex, but at least a 2.7 fold increase in TNF- α was measured in the (C) hippocampus in the EtOH/EtOH group compared with all other groups. No change in TNF-α was seen in (D) entorhinal cortex. *p < 0.05 compared to all groups.*

Table 4.5 Select Hippocampal Cytokine and Growth Factor Correlation Analyses

Cytokine	Parameter	Correlation Coefficient	P-value
TNF-α - EtOH/EtOH	Intoxication behavior	S=0.371	0.36
	Dose/Day	P= -0.544	0.16
	Total Dose	P= -.3545	0.39
	BEC	P=0.807	**0.02***
	Percent Weight Loss	P=0.610	0.11
	OX-42 immunoreactivity	P= -0.139	0.74
	Iba-1+ Cells	P= -0.372	0.36
BDNF-Con/EtOH	Intoxication behavior	S= -0.421	0.23
	Dose/Day	P=0.166	0.65
	BEC	P=0.166	0.64
	Percent Weight Loss	P=0.395	0.26
	OX-42 immunoreactivity	P=0.253	0.48
	Iba-1+ Cells	P=0.835	**0.003***
BDNF-EtOH/EtOH	Intoxication behavior	S=0.216	0.62
	Dose/Day	P= -0.149	0.73
	Total Dose	P= -0.144	0.73
	BEC	P= 0.298	0.47
	Percent Weight Loss	P= -0.473	0.24
	OX-42 immunoreactivity	P= -0.254	0.54
	Iba-1+ Cells	P=0.224	0.59

Table 4.5 Correlations were used to examine the relationship between cytokines versus immunohistochemical markers of microglial response and animal model data. TNF-α and BECs in the EtOH/EtOH group as well as the number of microglia and BDNF in the Con/EtOH group, both were correlated significantly.

Figure 4.7 TNF-α and BEC Correlation of EtOH/EtOH group

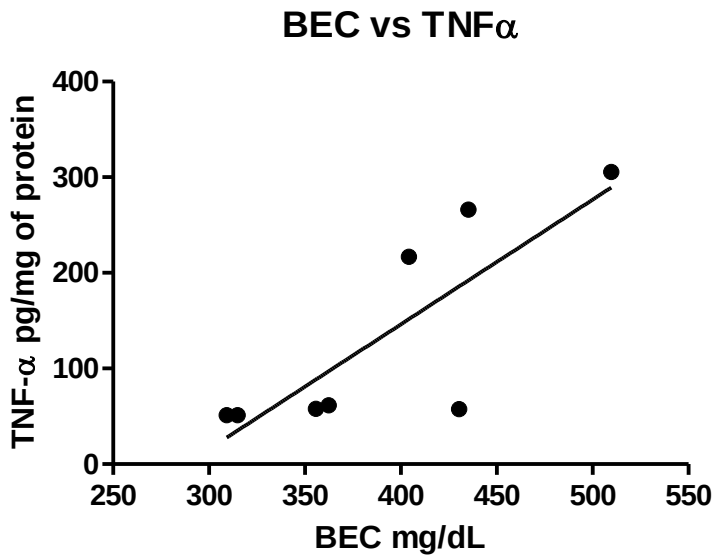


Figure 4.7 A positive correlation was observed between BEC and TNF-α concentration. Animals with BECs over 400mg/dL appear to have an increase in TNF-α.

Differential effects of treatment on BDNF concentrations

BDNF concentrations were assessed as a quick measure of the health of the neuronal environment in the hippocampus as BDNF is associated with neuronal cell survival (Lipsky and Marini 2007; Loeliger et al. 2008). A one-way ANOVA of BDNF concentrations indicated a significant effect of treatment in the hippocampus [$F_{(3,28)}$ =19.00, p<0.0001]. Post-hoc Tukey's tests indicated a 20% increase in BDNF concentration in the hippocampus in the EtOH/EtOH compared with all other groups (Figure 4.8). Consistent with data presented in chapter three (Figure 3.4), Tukey's test indicated Con/EtOH rats had decreased concentrations of BDNF compared to both the Con/Con and *ad libitum* group (Figure 4.8). Correlations between binge animal model data as well as markers of microglial activation were run versus BDNF concentrations for both the Con/EtOH and EtOH/EtOH group (Table 4.4). The estimated total number of

microglia [$P_{(10)}$ =0.835, p=.003] was correlated to BDNF concentrations only in the Con/EtOH group (Table 4.5; Figure 4.9).

Figure 4.8 Differential effects of Ethanol Exposure duration on BDNF

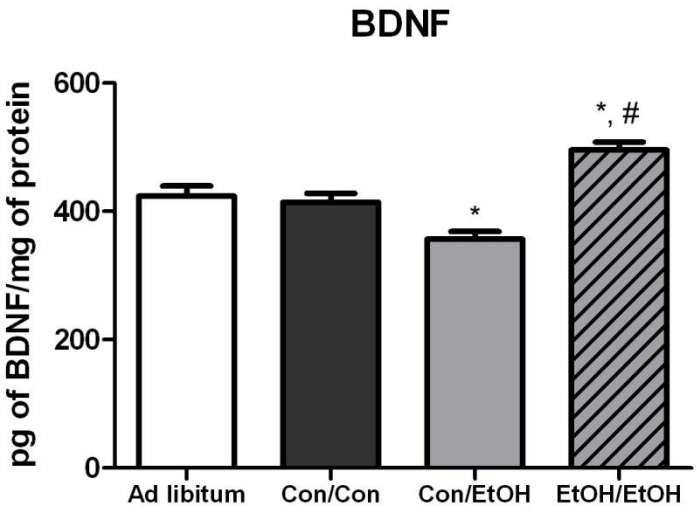


*Figure 4.8 Concentrations of BDNF were determined by ELISA in the hippocampus. BDNF was decreased by approximately 15% in Con/EtOH treated animals compared with Con/Con or ad libitum groups but increased by 20% of in the EtOH/EtOH groups. *p < 0.05 in relation to ad libitum and Con/Con group; # p < 0.05 in relation to Con/EtOH.*

Figure 4.9 BDNF and Stereological Estimates Correlation of Con/EtOH group

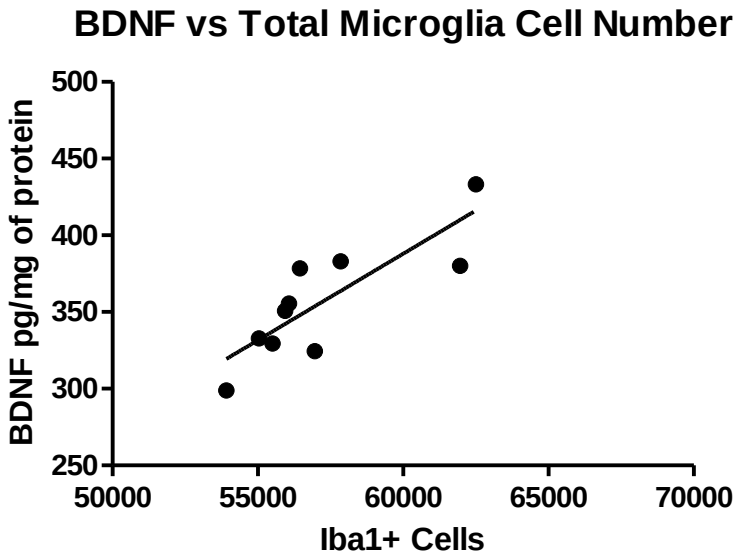


Figure 4.9 A positive correlation was observed between hippocampal estimates of microglia number and BDNF concentrations in the Con/EtOH. A decline in the number of microglia cells was related to decreases in BDNF concentrations.

DISCUSSION

During abstinence, the Majchrowicz model of alcohol-induced neurodegeneration results in microglia that are partially activated. Partially activated microglia can be involved in recovery mechanisms, but an alternative interpretation of partial microglial activation is that the microglia are primed. The studies herein further develop our understanding of what may occur within the alcoholic population by examining the effects of repeated binge exposure in a rodent model. Two criticisms of Majchrowicz model is that animals have prolonged periods of intoxication and that it's just one exposure period whereas human alcoholics drink in a more episodic nature such that there are periods of high BECs with but also periods of abstinence (Harford et al. 2005; Hunt 1993; White et al. 2006). Therefore, these experiments tested whether the partially activated microglia previously observed following four days of ethanol exposure were

primed and could be potentiated by a second insult of ethanol exposure. Increased OX-42 immunoreactivity as well as differential TNF-α production in the EtOH/EtOH group compared to the Con/EtOH group supports the major finding of these studies that a subsequent binge exposure can potentiate alcohol-induced microglial activation. Given that people often drink in an episodic binge pattern, potentiation in this model, with just two exposure experiences, suggests that chronic alcohol exposure may lead to even more dynamic microglial activation over time.

The first evidence of this differential response observed here was increased immunoreactivity of the OX-42 antibody. Ethanol exposure has been shown previously to cause increased OX-42 staining (Fernandez-Lizarbe et al. 2009; Marshall et al. 2013; Zhao et al. 2013). This study confirms those findings but furthers that work by showing that a second hit of binge ethanol exposure further increases OX-42 immunoreactivity. Increased OX-42 immunoreactivity is indicative of an upregulation of the CR3 receptor which can lead to increased phagocytic activity (Hynes 1992; Morioka et al. 1992; Robinson et al. 1986). Because the switch from low-grade activation to classical activation is CR3 dependent (Ramaglia et al. 2012), the differentially increased upregulation of CR3 caused by secondary ethanol exposure in the EtOH/EtOH group indicated that more classical signs of activation would also be present. However, despite the potentiation of the CR3 receptor density, no changes in ED-1 or OX-6 staining were seen following the second binge. The lack of ED-1 upregulation concurs with many other studies that do not show signs of phagocytosis following ethanol exposure (Marshall et al. 2013; McClain et al. 2011; Nixon et al. 2008). However, one recent but controversial report, due to the lack of animal recovery and mortality, has shown that more chronic ethanol exposure results in phagocytic, classical activation of microglia (Zhao et al. 2013). Furthermore, the OX-6 positive cells seen in one EtOH/EtOH animal, while an

anomaly in this study, may be a sign of a progression of activation from a low-grade to a more classical activation state as seen in other models with more prolonged intermittent exposure (Ward et al. 2009a).

Microglial activation not only causes changes in receptor density but also affects secreted cytokines (Carson et al. 2007), therefore hallmark pro- and anti-inflammatory cytokines were measured to understand the type of microglial activation associated with a second binge ethanol exposure. No change in IL-10 concentration was caused by ethanol exposure in either group within the hippocampus or entorhinal cortex. The lack of IL-10 response concurs with data presented in chapter two that during intoxication anti-inflammatory cytokines are not changed (Figure 2.6; Marshall et al. 2013). That same report showed an increase in IL-10 following seven days of abstinence (Marshall et al. 2013). Whether the normalized levels of IL-10 reported here are caused by secondary ethanol exposure or a byproduct of a transient increase in anti-inflammatory cytokines cannot be answered by the current experiments. However, the normalized levels of IL-10 does indicate that primed microglia progress are not secreting anti-inflammatory cytokines. Instead an increase in TNF-α, secreted by proinflammatory microglia, was seen in the EtOH/EtOH group compared with all other groups in the hippocampus. Although the methods used cannot directly tie changes in TNF-α concentration to microglia, the change in the cytokine profile, at minimum, suggests a proinflammatory state within the hippocampus caused by secondary ethanol exposure. An increased proinflammatory state may be reflective of the primed state of microglia observed following secondary ethanol exposure.

The differences in dose per day among the ethanol treated animals did not appear to have an effect as correlative studies looking at dose per day did not have an effect on microglia markers in either the Con/EtOH or EtOH/EtOH group. Moreover, the

total dose given to the EtOH/EtOH animals did not correlate to increases in any markers of microglial activation. This lack of correlation suggests the potentiation in OX-42 immunoreactivity in the EtOH/EtOH group were not due to a simple additive effect of alcohol. Correlation studies were also used to examine the upregulation of TNF-α observed in some but not all animals. Although upregulation of the CR3 receptor is associated with increased activation and the observed increase in microglia number could affect basal cytokine levels, the increase in TNF-α levels were not associated with either parameter. BECs, however, did have a significant relationship to TNF-α levels. The mechanism by which BECs could influence TNF-α expression was not determined in these studies, but higher BECs can alter the way ethanol is metabolized. At higher BEC, ethanol is metabolized within the brain more readily by CYP2E1 which is an alternative mechanism of increased ROS production in alcoholics (Haorah et al. 2008; Ronis et al. 1993; Zhong et al. 2012). An increase in ROS production caused by ethanol metabolism could also explain differences seen between these data and others that show more robust classical activation of microglia (Qin and Crews 2012b).

Data presented in chapter three showed that intoxication caused a decrease in microglia number. Similar to data described in chapter three, the Con/EtOH group had decreased numbers of microglia. This reduction may be due to degeneration or loss of microglia and dysfunction in both the hippocampus and entorhinal cortex. However, these experiments were conducted to determine whether primed microglia were still susceptible to the decreases associated with intoxication with a second binge event. The EtOH/EtOH group actually showed an elevated number of microglia within the hippocampus compared with the control groups and Con/EtOH animals. These data agree with increased microglia numbers seen in human alcoholics (He and Crews 2008). However, interpreting these data is complicated by the fact microglia begin to proliferate

in the hippocampus 48 hours after the last dose of ethanol (McClain et al. 2011; Nixon et al. 2008). This proliferation results in increased microglia at the time that the second binge paradigm began (Marshall et al. 2013), but the lack of decrease does allude to the idea that primed microglia may not be as susceptible to microglial dysfunction or degeneration. Unlike in the hippocampus, microglia proliferation in the entorhinal cortex does not cause an increase in cell number at the time in which the second exposure starts (Marshall et al. 2013). The similar number of microglia observed among the Con/EtOH and EtOH/EtOH group in the entorhinal cortex indicates that despite having a more robust response (increased OX-42) that microglia are still susceptible to the damaging effects of alcohol during intoxication. The differential susceptibility of brain regions may be due to differences in the type of activation between the two regions as the hippocampus was shown to have more classical signs of activation (TNF-α). Increases in microglia, such as the sustained increase in hippocampal microglia observed in the EtOH/EtOH group, have been described as causative in increased neuroinflammatory activity in other neurodegenerative disorders (Frank-Cannon et al. 2009). Importantly, increased microglia in the hippocampus support the theory that repeated ethanol exposure causes a differential response in microglia primed by previous exposure.

A more chronic model of alcohol exposure using intragastric exposure also suggests that microglia classical signs of activation, but there is controversy regarding interpretations of the data due to the health of animals and their lack of recovery from the binge model (Zhao et al. 2013). The intragastric gavage method used in this model can be stressful to animals and results in weight loss due to caloric restriction (Balcombe et al. 2004; Sharrett-Field et al. 2013a). Both stress and reduced caloric intake can alter microglial activation (Loncarevic-Vasiljkovic et al. 2012; Sugama 2009; Sugama et al.

2009). To confirm that changes in microglial activation and cell number were due to ethanol and not other factors associated with the repeated gavage, a group with *ad libitum* access to food and water was assessed and compared with the Con/Con group. The measures of activation used to assess the microglia were not different in any aspect between the *ad libitum* group and the Con/Con group despite the weight loss caused by intragastric gavage. Furthermore, weight loss did not correlate with any measure of microglial activation and is therefore probably not a factor in the changes between Con/EtOH or EtOH/EtOH animals.

Upregulation of indices of microglial activation and number coincide with changes in the concentration of the growth factor, BDNF. The pattern seen in hippocampal BDNF alterations mimics the changes seen in microglia cell number and activation. In the Con/EtOH group, BDNF was decreased as cell number decreased; however when microglia are more activated and numbers are increased, EtOH/EtOH treated animals had an increase in BDNF concentration. Increases in BDNF may promote cell survival (Lipsky and Marini 2007; Loeliger et al. 2008). Because BDNF is secreted by astrocytes, neurons, and other cells within the CNS (Bejot et al. 2011; Lau and Yu 2001), the methods used cannot definitively state that changes in BDNF concentrations are due to microglia. However, in the Con/EtOH the number of microglia was positively correlated to BDNF concentration. The correlation between microglia loss and BDNF reduction supports the idea that microglial dysfunction and subsequent loss of trophic factors is a concern in alcoholic brain damage. The lack of correlation in the EtOH/EtOH group may be due to increased TNF-α concentration on the release of BDNF from astrocytes, as astrocytes are also more reactive in abstinence from alcohol exposure (Kelso et al. 2011; Saha et al. 2006). Regardless of the source, this increased BDNF indicates that

both in proinflammatory cytokines and proneurogenic growth factors are present within the milieu of the hippocampus after a second binge ethanol treatment.

Whether the alcohol-induced microglial activation shown within affords neuroprotection or leads to increased damage cannot be determined from these studies. At a glance, it would appear that increased microglial activation especially with increased secretions of TNF-α would be detrimental to the neuronal environment as shown in other neurodegenerative diseases (Block and Hong 2005). However, an acute initial microglial response is necessary for recovery and the removal of neuronal debris (Badoer 2010; Nimmerjahn et al. 2005; Streit 2005). The loss of microglia during intoxication was discussed in chapter 3 and was purported to be associated with a loss of homeostatic properties of microglia; however, in the hippocampus microglia number remain elevated in response to damage. This elevation in cell number may suggest that microglia are actually responding more appropriately to the alcohol-induced brain damage. Moreover, studies with more chronic binge alcohol exposure produced phagocytic microglia and proinflammatory cytokines that were transient and present mainly during intoxication (Zhao et al. 2013). The transient nature of this response indicates that microglia are actually responding appropriately to neuronal damage. If the upregulation of TNF-α seen in the EtOH//EtOH group is only transient it would indicate that response of microglia is only induced by neuronal injury and that activation occurs as a rehabilitative event to restore homeostasis. For example, as described earlier, acute TNF-α upregulation can induce BDNF production in astrocytes and therein afford neuroprotection (Saha et al. 2006).

The potentiated microglia activation seen in this double binge AUD model suggests that the microglial response can be altered by ethanol alone. Whether this increased response causes microglia to respond more appropriately to noxious stimuli or

if it makes the brain more susceptible to ongoing neuroinflammation cannot be determined by these experiments. However, because microglia have the capacity to maintain low grade activation for extensive periods following alcohol exposure (Marshall et al. 2013; Obernier et al. 2002b), the episodic nature of binge drinking may lead to a cycle of repeated priming activity within individuals suffering from an AUD. Furthermore, instances of systemic inflammation and ROS production may act to perturb already primed microglia (Cunningham 2013; Cunningham et al. 2005). These data do not conclusively indicate microglial activation as a source of alcohol-induced neurodegeneration, but this study does show that repeated ethanol exposure potentiates microglial activity. The primed, persistent nature of microglia following alcohol-induced neurodegeneration observed in this model may still be a source of neurodegeneration in human alcoholics especially when other neuroimmunomodulatory factors are present and the AUD is more chronic in nature.

OVERALL CONCLUSIONS

Review

Alcoholism is a chronic disease, which permeates various aspects of society. Chronic ethanol exposure leads to neuroplastic changes that drive the development of an AUD. Elucidating these neurobiological changes has led to the development of therapies that ameliorate craving and/or the rewarding effects of alcohol, but currently no therapies specifically treat the neurodegeneration caused by excessive alcohol consumption. Alcohol-induced neurodegeneration is associated with cognitive deficits that compromise the executive function and the working memory. These cognitive deficits caused by neuronal loss can perpetuate the seeking of alcohol and therefore have been hypothesized as contributing to the development of an AUD (Crews 2012; Crews et al. 2011). Like other neuroadaptations within AUDs, elucidating the mechanisms that lead to alcohol-induced neurodegeneration may be a novel therapeutic target for the treatment of alcoholism.

Neuroinflammation is a key factor in many neurodegenerative diseases like Alzheimer's and Parkinson's Diseases and recently has been proposed as a mechanism of alcohol-induced neurodegeneration. The current understanding of alcohol's effects on the neuroimmune system in alcoholics does not definitively indicate that neuroinflammation occurs within AUDs; however, they do suggest that alcohol alters the neuroimmune system. One such effect is the activation of microglia. Microglial activation has long been considered a hallmark of neuroinflammation, but understanding the dynamic nature of microglia within the context of a disease is crucial to understanding how they may be involved with neurodegeneration and/or homeostatic recovery mechanisms. Therefore, these studies examined microglial activation within an AUD model of alcohol-induced neurodegeneration looking at both the initiation and level of

activation as well as the ability of persisting microglia activation to exacerbate the neuroinflammatory events.

Aim 1: Determine the phenotype of microglia reactivity following binge ethanol exposure in the Majchrowicz model of an AUD (Chapter 2).

The hypothesis that binge ethanol exposure induces low-grade microglia activation was supported by experiments herein. Increased expression of CR3 (OX-42) and TSPO as well as anti-inflammatory cytokines without an increased immunoreactivity of ED-1 or OX-6 suggest that acute binge ethanol exposure does not elicit classically activated microglia but shows signs of partial activation. Specifically, our data indicates that microglia are elicited to stage2b of the Raivich scale. The lack of classically activated microglia in conjunction with no BBB disruption therefore does not meet the criteria for neuroinflammation.

Aim 2. Determine the earliest indices of microglial activation in the Majchrowicz model of an AUD (Chapter 3).

The hypothesis that the initial microglial response occurs subsequent to indications of neurodegeneration was supported by increased binding of [^{3}H]-PK-11195 after two but not one days of exposure. Albeit not a part of the original hypothesis goals, an unexpected discovery of decreased microglia number and evidence of dystrophy suggests a type of microglial dysfunction in a second population despite activation in some microglia.

Aim 3. Determine if alcohol-induced microglia reactivity following the Majchrowicz model is “primed” (Chapter 4).

The hypothesis that a second binge exposure would potentiate the microglia response seen in recovery from the Majchrowicz model was supported, but not robustly. However, functional and immunohistochemical assessments of activation differed on whether the potentiation induced classical signs of activation. OX-42 immunoreactivity was more robust following two binge ethanol exposures paradigms than with a single binge paradigm, but neither ED-1 nor OX-6 was increased by secondary binge ethanol exposure. However, ELISA studies showed that TNF-α was increased only after the second binge treatment. This change in response suggests that the initial response of microglia in the Majchrowicz model is primed.

Discussion

The purpose of this dissertation was to investigate the effects of ethanol on microglia within the context of an AUD model known to cause neurodegeneration. To synthesize the findings within this dissertation within a larger scheme, the effects of ethanol on microglia will be considered within either the context of intoxication or abstinence. Microglial responses were studied with an emphasis on the phenotype of microglial activation and the initiation and duration of activation. Together these characteristics of microglial activation can be used to infer the contributions of microglia in alcohol-induced brain damage. The data reported here suggest at least three states of microglia that may contribute to either brain damage or recovery from damage: dysfunctional, neurotrophic, and primed.

Microglial Dysfunction

In chapters two and three, the data showing increases in CR3 and/or TSPO without signs of classical activation agree that microglia are activated to a low-grade state in response to alcohol-induced neuronal damage. This alternative activation state of microglia is not indicative of the classical pathways of microglia leading to

neurodegeneration (Carson et al. 2007). However, the reductions in the number of microglia and the appearance of dystrophic microglia reported in chapter three indicate that despite microglial activation that a subset of microglial cells is dying. As previously discussed, these findings suggest that binge ethanol exposure could disrupt the normal function of the neuroimmune system. Following damage, microglia provide immediate neuronal support by promoting anti-inflammatory mechanisms, secreting neurotrophic factors, and/or through proinflammatory activation removing cellular debris (Streit 2002a). The reduction in the number and/or function microglia could affect these recovery mechanisms afforded by microglia and be a source of damage. This hypothesis is an alternative source of neurodegeneration to the typically described neuroinflammatory pathways that are thought to be associated with alcohol abuse (Crews 2012; Crews et al. 2011; Streit et al. 2009). While this dissertation does not specifically look into the mechanisms by which dysfunction of microglia may cause neurodegeneration, the role of microglia in neurogenesis and glutamate reuptake are at least two ways in which a dysfunctional neuroimmune response could lead to alcohol-induced neurodegeneration.

It has been shown that a deficiency in microglia disrupts neurogenic processes and reduces recovery from neuronal damage (Wainwright et al. 2009). Activated microglia migrate following neuronal damage and secrete cytokines and growth factors associated with supporting the neurogenic niche in the hippocampus (Neumann et al. 2006). However in this AUD model, the hippocampal microglia “nursing” response to increase neurotrophic factors such as IL-10 and TGF-β was not observed immediately in conjunction with signs of neurodegeneration (Hayes et al. 2013; Streit 2002a; Takayama and Ueda 2005). The reduction in microglia and the lack of neurotrophic support seen herein during acute intoxication may be involved with the interruption of neurogenic seen

during intoxication (Morris et al. 2010a; Nixon and Crews 2002). In particular, decreased levels of BDNF seen during intoxication would decrease newly born cell survival and provides indirect evidence of the potential role of microglia in the reduction of adult neurogenesis observed in this AUD model (Lipsky and Marini 2007; Loeliger et al. 2008; Mitchell 1999).

The relationship between microglia and glutamate concentration was not examined in this study but represents another way in which a reduction in microglia number could potentially affect neuroadaptations seen following alcohol exposure. Activated microglia upregulate GLT-1 leading to the amelioration of excessive glutamate levels (Persson et al. 2005; van Landeghem et al. 2001). However, the reduction in microglia number in damaged regions may be a source of disrupted uptake of glutamate. Since alcohol withdrawal is associated with excess glutamate concentrations, the transient decrease in microglia number observed during intoxication in chapter three may be a factor in glutamate excitotoxicity. Specifically, the idea that alcohol prevents microglial reuptake of glutamates provides further alludes that the loss of microglial may be a source of neurodegeneration (Gras et al. 2003). Activated astrocytes have been shown to increase their glutamate uptake in response to ethanol exposure (Miguel-Hidalgo 2006; Mulholland et al. 2009; Smith 1997), but the potential role of microglia in recovery from glutamate excitotoxicity caused by ethanol exposure remains elusive.

Neurotrophic Microglial

Fortunately, neuronal deficits caused by binge alcohol exposure are partially recovered during abstinence (Zahr et al. 2010b). This recovery in the hippocampus is thought to be afforded in part to reactive neurogenesis (Crews and Nixon 2009; Nixon and Crews 2004; Zahr et al. 2010b). Intriguingly, the level of microglia activation observed during abstinence in chapter two suggests that microglia may participate in

recovery during abstinence by promoting neurogenesis (Kohman and Rhodes 2013; Varnum and Ikezu 2012). The increase in the proneurogenic cytokines IL-10 and TGF-β reported in chapter two occurs concurrently with evidence of neuroprogenitor cell proliferation (McClain et al. 2013; Nixon and Crews 2004). However, coincident timing alone is not enough to distinctly determine whether these cytokines are involved with neuronal proliferation or the survival of neurons, but evidence from other fields with reactive neurogenesis suggest that such anti-inflammatory cytokines promote the survival and or proliferation of newly populated cells (Battista et al. 2006; Ekdahl et al. 2009; Kiyota et al. 2012). A direct relationship between partially activated microglia and reactive neurogenesis in this AUD model is a point of interest and should be considered in the future studies.

Primed Microglia

Partially activated microglia can also be primed and exacerbate the neuroimmune response upon subsequent insults (Bilbo and Schwarz 2009; Norden and Godbout 2013). The potential of partially activated microglia to exacerbate the neuroimmune reaction is particularly of concern given the enduring nature of microglial activation. As described in chapter two, CR3 (OX-42) was upregulated for at least twenty-eight days ethanol exposure (Marshall et al. 2013). Independent studies agree that ethanol-induced microglial activation persists into protracted abstinence (Obernier et al. 2002b). This prolonged activation of microglia led to the studies performed in chapter four determining whether the microglia response was primed and would react differently to other immunological stimuli. Using ethanol as both the initial and secondary neuroimmunomodulator, data collected suggested that alcohol exposure does result in a primed state of microglia such that chronic alcohol exposure may result in more robust responses over time. These studies do not indicate whether this potentiated

neuroimmune response endures into abstinence and would truly create an environment of chronic neuroinflammatory state. Chronic microglial activation would perpetuate neurodegenerative cell signaling cascades. However, examinations of microglia following repeated bouts of binge treatment imply that the proinflammatory state is only transient (Zhao et al. 2013). The transient proinflammatory response observed following repeated binge paradigms may actually be involved in homeostasis by removal of cellular debris as phagocytic microglia were seen in other models or by inducing neurotrophic factor release from other cell types.

Although these studies show specifically that ethanol alone can exacerbate a primed microglial response, others have shown that alcohol-induced microglial activation can be exacerbated by systemic inflammation (Qin et al. 2008). It is important in considering the implications of the alcohol-induced primed microglia state seen herein that the systemic immune system can exert effects on the CNS neuroimmune response making it more susceptible to damage (Drake et al. 2011; Murray et al. 2013). This influence would be particularly critical in complicated alcoholics who may have liver damage or a comprised BBB (Crews et al. 2011; Qin et al. 2008; Qin et al. 2013). If immune challenge in the peripheral organ systems initiated an immune response, alcohol-induced primed microglia may surmount a response in the absence of neuronal damage. This response to noxious stimuli from the periphery may then cause damage to healthy tissue.

Alcohol-Induced Microglial Neuroadaptation

The differential response of microglia with respect to duration of exposure as well as in abstinence or intoxication is comparable to other neuroadaptations having differential outcomes with acute versus chronic exposure and with intoxication versus withdrawal/abstinence (Vengeliene et al. 2008). For example, whereas acute ethanol

inhibits the glutamate receptor, chronic ethanol causes neuroplastic changes that result in an increase in glutamatergic signaling over time (Vengeliene et al. 2008). Likewise, the data presented imply that acute alcohol intoxication initially inhibits or at least disrupts normal neuroimmune function, but chronic ethanol exposure results in a more proinflammatory response. Figure 5.1 summarizes the response of microglia seen within. The differential response between acute and chronic ethanol exposure aligns with the idea that ethanol causes neuroplastic changes in the neuroimmune system. Because ameliorating alcohol-induced neuroadaptations has proven to be effective in treating AUDs, so may controlling the neuroimmune response of alcoholics may afford neuroprotection. The neuroimmune system and particularly microglial activation remains a target of interest in reducing the neurodegeneration associated with AUDs.

Figure 5.1 Microglial Morphology & Function in an AUD Model

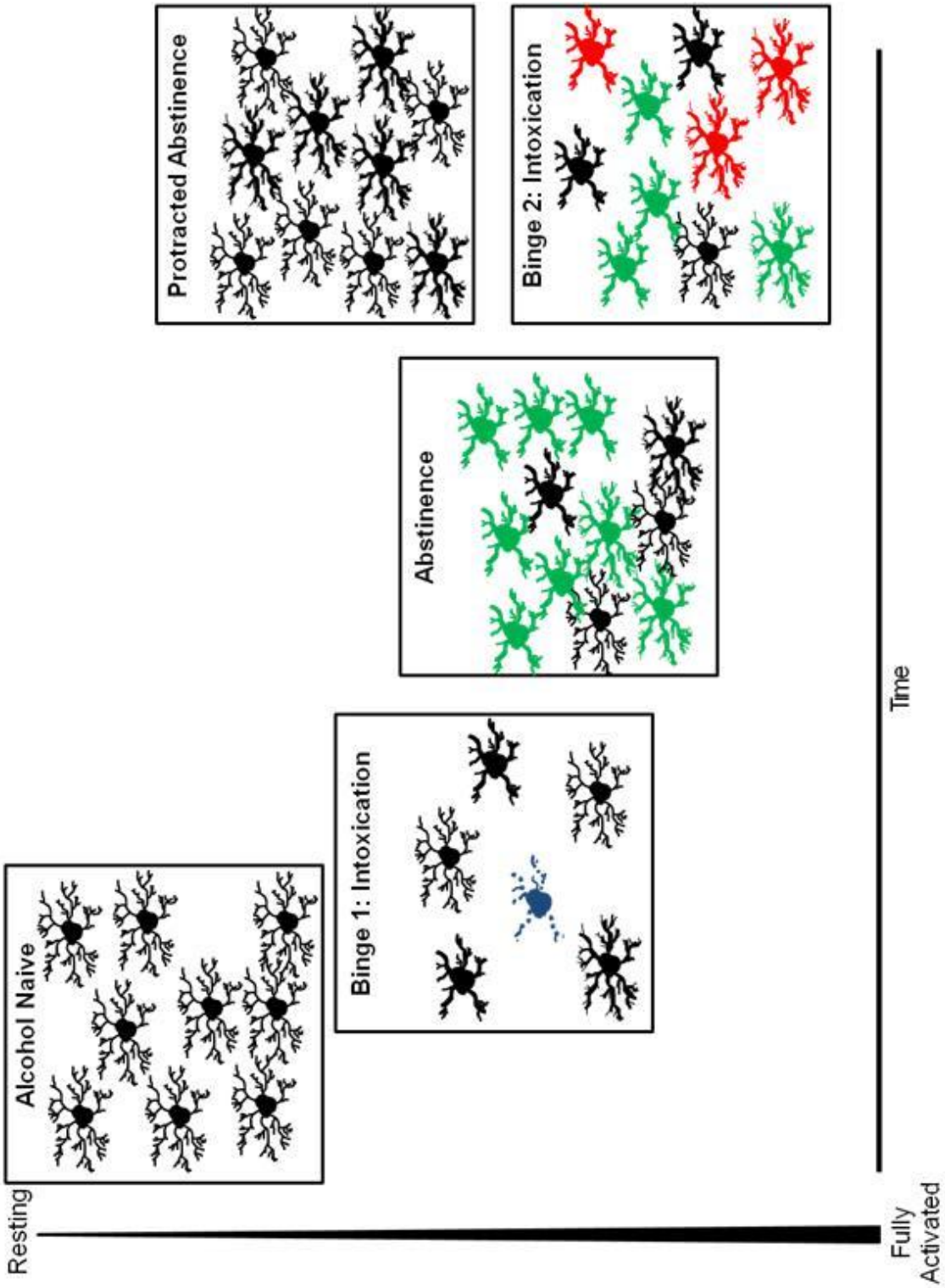


Figure 5.1 A depiction of the microglial response seen within these studies. Binge treatment inhibited microglial function, but during abstinence microglia become partially activated and secrete neurotrophic factor. The persisting, primed nature of this activation however exacerbates microglia activation by a second exposure. Blue represents dystrophic microglia, thickened cells represent a morphological or protein expression change, thickened green cells represent the emergence of anti-inflammatory cytokines, and thickened red cells represent the secretion of proinflammatory cytokines.

Limitations & Future Studies

One of the limitations of these studies was the inability to directly tie the changes in microglia response to recovery. In chapter two, the data set suggests that microglia would be involved in recovery during abstinence by promoting neurogenesis. In order to directly look at this relationship would require knocking down or inhibiting the microglial response. However, pharmacological treatments for inhibiting partially activated microglia are limited as therapies are generally directed at inhibiting classical, full activation of microglia or at promoting the alternative activation. For example, standard neuroimmunomodulators such as non-steroidal anti-inflammatory drugs and minocycline both target proinflammatory microglia and can promote the alternative activation state (Kobayashi et al. 2013; Lee et al. 2010; Wang et al. 2012a). Moreover, these agents have not been shown to reduce partial activation. However, one way to determine to whether partially activated microglia observed in abstinence from alcohol exposure are involved with reactive neurogenesis *in vivo* is to use an alternative model with transgenic CCR3 deficient mice such as used in the facial axotomy model (Wainwright et al. 2009). The facial axotomy model has shown that axonal regeneration is dependent upon the function of microglia using these mice. As such CCR3 deficient mice may be a useful tool in understanding the contributions of microglia to reactive neurogenesis, but this

model would require elucidating a new timeline of neuronal and glial events that has already been done in the rat model.

A different mechanism by which microglia may afford neuroprotection is the upregulation of GLT1 and the removal of glutamate (Persson et al. 2005; van Landeghem et al. 2001). This theory deserves exploration but may not be feasible in the current model as glutamate excitotoxicity has not been observed in the Majchrowicz model (Rudolph et al. 1997).

Another limitation of these studies is that naive animals are given a bolus of ethanol in adulthood. This type of ethanol exposure does not necessarily reflect the human condition as people generally experiment with lower concentrations of ethanol during adolescence before consuming the neurotoxic levels used within these experiments (Guilamo-Ramos et al. 2004; Nixon and McClain 2010). While the perfect model of alcoholism would include preconditioning with lower concentrations prior to the bolus, the model of alcohol-induced degeneration use in these studies do at least reflect the response of microglia to alcohol-induced neuronal damage. Furthermore, even moderate concentrations of alcohol result in modulations to the neuroimmune system with in vivo with chronic exposure (Ehrlich et al. 2012) and even acutely in culture (Collins et al. 2010; Fernandez-Lizarbe et al. 2008). The ability of ethanol to affect this system at lower concentrations suggests that in the development of an AUD experimentation with ethanol would also result in a microglial response that may progress and play a role in the neuroadaptations within the neuroimmune response.

All of these studies were done in males despite the fact that AUD-associated neurodegeneration is also seen in females. While alcohol-induced degeneration may be more prevalent in males, females actually are thought to be more susceptible to damage (Hommer 2003; Prendergast 2004; Sharrett-Field et al. 2013b). The model used in these

studies does not show differential damage between male and female rats, but others have shown increased susceptibility in the neuroimmune response to alcohol in models of AUDs (Alfonso-Loeches et al. 2013). Understanding the role of microglial activation in female rats would further our understanding of the neuroimmune response and its relationship to neurodegeneration and recovery in the human population.

Final Comments

The current dissertation work delineates the response of microglia to ethanol exposure in the most comprehensive manner to date looking at the level of activation over a timecourse in an AUD model. The results indicate that the microglial response changes with respect to the duration of exposure as well as whether the observation is during intoxication or abstinence. While initial intoxication may suppress or disrupt the neuroimmune response, during abstinence the microglia response recovers. The phenotype of activations suggests that the microglia would be neurotrophic to the environment. However, a second binge exacerbates the microglial response due to the persisting primed microglia from the initial alcohol insult. These data support the idea that the function of microglia are affected by alcohol and that repeated exposure may cause a neuroplastic change in the microglial response. Pharmacological interventions that promote the neurotrophic mechanisms of microglia while simultaneously limiting their detrimental effects may prove efficacious in recovery from alcohol-induced neurodegeneration.

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EDUCATION AND TRAINING

08/2008-Present	University of Kentucky, Doctoral Candidate, Pharmaceutical Sciences
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08/2004-05/2008	University of Florida, B.S. Zoology

RESEARCH AND TEACHING EXPERIENCE

01/2009-Present	Research Assistant, University of Kentucky, Dr. Kimberly Nixon, Principle Investigator,
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06/2007-06/2008	Research Assistant, University of Florida, Dr. Adriaan Bruijnzeel
09/2004-05/2007	Research Intern, University of Florida, Dr. Joana Peris
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PROFFESSIONAL HONORS

2012	Frontiers in Addiction Research NIDA Mini-Convention Travel Award
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2008- 2010	Lyman T Johnson Fellow, *University of Kentucky*.
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PUBLICATIONS

McClain JA, Morris SA, **Marshall SA**, Nixon K. *Ectopic hippocampal neurogenesis in adolescent male rats following alcohol dependence.* Addiction Biology. Epub ahead of print PMID: 23844726

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Marshall SA and Nixon K (2010). *Selective changes in hippocampal cytokine production following binge ethanol exposure.* Bluegrass Society for Neuroscience

Marshall SA and Nixon K (2010). *Selective changes in hippocampal cytokine production suggest partial activation of microglia following binge ethanol exposure.* Research Society on Alcoholism

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Liput DL,.Hammell DC, **Marshall** S**A**, Stinchcomb AL, and Nixon K (2009). *Attenuation of Alcohol-Induced Neurodegeneration Via Transdermal Delivery of Cannabidiol.* Research Society on Alcoholism